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# METABOLISM OF AROMATIC NITRO COMPOUNDS.

## 1. ESTIMATION OF DIAZOTISABLE AMINES IN RATS' AND HUMAN URINE AFTER INTAKE OF 2.4.6–TRINITROTOLUENE.

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*(Accepted for publication 24th November, 1944.)*

Dale *et al.* (1921) had shown that a small percentage of 2.4.6–trinitrotoluene (T.N.T.) injected subcutaneously into rabbits was excreted in the urine in the form of an unidentified aromatic aminocompound; the other product they obtained was the 4–azoxy compound. Early in 1942, Mr. J. W. Legge, at this Institute, observed that large amounts of diazotisable amines were excreted by the rat after ingestion of T.N.T. After acid hydrolysis, diazotisation, destruction of excess nitrite and coupling with dimethyl-α-naphthylamine or the reagent of Bratton and Marshall (1939) the azodyes were determined spectrophotometrically or colorimetrically. Similar observations have been made meanwhile by American and English authors, and a number of methods for the estimation of diazotisable amines derived from T.N.T. have been published (Westfall and Smith, 1942; Pinto and Wilson, 1943; Snyder and v. Oettingen, 1943; Channon *et al.*, 1944). We have delayed publication until we had ascertained by metabolism experiments on rats and on human volunteers that the amount of diazotisable amines is always roughly proportional to the T.N.T. intake (*c.f.* the second paper of this series), and until the quantitative estimation could be put on a sound basis by isolation of the major reduction products of T.N.T. from the urines of rat and men.

Pinto and Wilson (1943) boil the urines with hydrochloric acid and remove the amino compounds from the ammoniacal urines containing some calcium hydroxide, by continuous ether extraction. They then reduce in dilute sulphuric acid with titanous chloride, destroy its excess with ferric alum, diazotise and couple with dimethyl–α–naphthylamine. The dye is determined photometrically or colorimetrically with a filter of maximum transmission at 524 mμ against a standard prepared from T.N.T. in similar manner. This method is laborious since ether extraction cannot be avoided. We can confirm the observation of the authors that urine interferes with the colour development after reduction with titanous chloride, but this is not so with the amino compounds present in unreduced urine. Since we found that the major T.N.T. reduction products present in the urines of men and rats give azo-dyes of a similar strength of absorption, there is no need for this reduction. The advantage of reducing the mixture of monoaminodinitro and diaminonitro compounds to triaminotoluene is spurious, since 5–nitro–*m*–phenylene diamine (Lemberg and Callaghan, 1944) and amino nitro cresols occur in rats' urine and do not yield triaminotoluene on reduction. Moreover, we have convinced ourselves that it is impossible to extract the diazotisable amines quantitatively by ether from urines after acid hydrolysis (cf. the third paper of this series).

The latter criticism also applies to the method of Snyder and v. Oettinger (1943), who extract the acid-hydrolysed urines with ether after neutralization with sodium bicarbonate, wash the ether extract with sodium carbonate, diazotise and couple with α–naphthylamine and determine the dye colorimetrically in a toluene extract with 4–amino–2.6–dinitrotoluene as a standard. In this method the diazotisable aminonitrocresols are partly lost in the bicarbonate washings.

The method of Westfall and Smith (1942), Channon *et al.* (1944) and our methods described below are based on the same general principle, i.e. diazotisation and coupling with the Bratton-Marshall reagent directly in the urines. This reagent is superior to dimethyl–α–naphthylamine. The methods differ in important details which will be discussed below.

[1] This work was supported by grants from the National Health and Medical Research Council of Australia.

We describe spectrophotometric methods for the estimation of diazotisable amines in the urines of rats and men. These were primarily based on the properties of 2.4–diamino–6–nitrotoluene, one of the major constituents of the T.N.T. reduction products in rats' urine, and of 4–amino–2.6–dinitrotoluene, one of the major constituents in human urine (cf. the third paper). Later investigation showed that the properties and constants of other amino compounds varied somewhat more widely, but the error introduced by the use of the constants derived from the first two compounds was hardly greater than the experimental errors. A simplified colorimetric estimation for human urines is also described.

## EXPERIMENTS.

*Spectrophotometric Estimation of Diazotisable Amines in Rats' Urine.*

To a measured volume of the urine conc. hydrochloric acid is added drop by drop, until the urine is just acid to congo paper. The volume is measured again and one-fiftieth of this volume of conc. hydrochloric acid is added. An aliquot is heated in a test tube placed in a beaker with boiling water for one hour and diluted to the original volume with water.

Reagents: 0·1 p.c. sodium nitrite in water; 0·5 p.c. ammonium sulphamate in water; 0·2 p.c. N–(1–naphthyl) ethylene diamine dihydrochloride in water; 1 p.c. hydrochloric acid.

To 1 ml. of acid-treated urine add 3 ml. 1 p.c. HCl and 1 ml. water. Add with good mixing 1 ml. of nitrite, 15 seconds afterwards 1 ml. of sulphamate and 30 seconds afterwards 1 ml. of the amine. Allow to stand for 2–3 hours.

Blank: 1 ml. of acid treated urine + 3 ml. 1 p.c. HCl + 4 ml. water.

The extinctions of the unknown and of the blank are determined in a Hilger Visual Absorption Spectrophotometer at 530 mμ in tubes of 1–4 cm. length. For urines containing very large amounts of diazotisable amines it is advisable to use only 0·5 ml. of urine.

If d is the dilution of urine by washwater (usually about 2) and acid treatment, and $\epsilon_u$ and $\epsilon_b$ the extinction coefficients of unknown and blank in 1 cm. layer, the content of diazotisable amine (expressed as μg. T.N.T. per ml.) in the urine is $\frac{10{,}000\ d(\epsilon_u - \epsilon_b)}{140}$. Multiplied by the volume of urine in ml. this gives the daily excretion of diazotisable amines.

Normal rats' urine contains diazotisable amino compounds. The excretion of these has to be determined for a number of days before dosing and to be subtracted in order to obtain the daily excretion of T.N.T. in the form of diazotisable amines.

The figure 140 is derived from the extinction of 2.4–diamino–6–nitrotoluene added to normal rats' urine and diazotised and coupled in the same manner. It is inadvisable to carry out the blank with additions of nitrite and sulphamate, omitting only the coupling reagent, since the diaminonitro compounds present in rats' urine develop a strong yellow colour on diazotation which disappears on coupling.

Table 1 shows that most of the colour is developed during the first 15 minutes. The maximum is reached after 2–3 hours and no significant decrease is found for 24 hours.

TABLE 1.

*Colour development.*

| 15 mins. | 45 mins. | 3 hours. | 24 hours. |
|---|---|---|---|
| 94 p.c. | 98 p.c. | 100 p.c. | 98 p.c. |
| 77 p.c. | 94 p.c. | 100 p.c. | 100 p.c. |
| 93 p.c. | 98 p.c. | 100 p.c. | 99 p.c. |

If the addition of the reagents to the urine is carried out as in the estimation of sulphonamides by Bratton and Marshall (1939), with 3 minutes standing after the addition of nitrite and 2 minutes waiting after the addition of sulphamate, lower values are found (between 60 p.c. and 90 p.c.. in average 79 p.c.) than with the method described above (cf. also Pinto and Fahy, 1942). This decrease is mainly due to the action of excess nitrite (cf. Table 2) and to a minor degree to the decomposition of the diazonium compound.

We recommend waiting 30 secs. after adding sulphamate, as this regularly suffices for complete destruction of nitrite. Nitrite reacts with the Bratton-Marshall reagent with the production of a purple dye, which is unstable and rapidly bleached, but adds a small amount to the absorption at 530 mμ.

TABLE 2.

*Effect of standing after addition of nitrite or sulphamate.*

| Waiting after addition of nitrite. | | | Waiting after addition of sulphamate. | | | | |
|---|---|---|---|---|---|---|---|
| | ε | | | ε | | | |
| | 1. | 2. | | 1. | 2. | 3. | 4. |
| 15 secs. | 2·72 | 2·31 | 15 secs. | 2·70 | 2·28 | 0·94 | 0·67 |
| 3 mins. | 2·35 | 1·94 | 2 mins. | — | — | 0·98 | 0·75 |
| 15 mins. | 2·00 | 1·59 | 5 mins. | 2·65 | 2·11 | 0·82 | 0·67 |
| | | | 15 mins. | 2·25 | 1·85 | — | — |

The effect of excess nitrite is due to its action on the diazonium compound of the diamino nitro compounds. Both 2.4–diamino–6–nitrotoluene and 5–nitro–*m*–phenylene–diamine give considerably higher extinction with addition of the reagents in rapid succession, while no such effect can be observed with the dinitrotoluidines. The effect is greater with the urine of rats to which T.N.T. has been given than with the urine of munition workers, since the former contains a far greater percentage of diaminonitro compounds. The mode of diazotisation and coupling has no influence on the extinction of diazotisable amines in normal urines.

The addition of larger amounts of nitrite, sulphamate or coupling reagent does not alter the results. The concentration of hydrochloric acid must be above 0·3 p.c. for rats' urine and above 0·6 p.c. for human urine before the addition of nitrite. If the acid concentration is too low, brown colours are obtained and addition of hydrochloric acid afterwards fails to restore full colour development. Above this concentration variation of HCl concentration was found to have no effects on the extinction in rats' urines, except that with unusually high amine concentration a tendency of the dye hydrochloride to precipitate was observed in solutions containing 5 p.c. HCl. We, therefore, use a concentration of about 0·2 N HCl which also gives maximal extinctions with diamino nitro compounds isolated from rats' urine.

Diazotisation at 0° C. did not give as high extinction as at room temperature in the manner described, but gave almost the same values, when the addition of reagents was carried out as in the Bratton-Marshall method.

*Estimation in Human Urine.*

Some human urines turn very dark on heating with dilute hydrochloric acid at 100° C. We therefore carry out the hydrolysis at room temperature allowing the acidified urines to stand for at least 24 hours. The values for diazotisable amine obtained in this way are only slightly lower than those obtained after boiling with acid. Normal human urines occasionally give a blue-green colour with the reagents, which contributes little to the extinction at 530 mμ, but may interfere with the colorimetric estimation of urines containing small amounts of T.N.T. derivatives. The chromogen disappears on allowing the acidified urines to stand for several days.

The formula given above was used for the spectrophotometric estimation. It will be seen below that the specific extinction of 4–monoamino–2.6–dinitrotoluene under the conditions of the estimation is slightly higher than 140, but this is counterbalanced by the presence of other derivatives which give dyes with lower extinction.

For the colorimetric estimation diazotisation and coupling are carried out as in the spectrophotometric method except that the final volume is made up to 10 ml. with alcohol. This gives a somewhat less bluish tint of colour. Standard: 5 ml. of 10 p.c. $CoSO_4.7H_2O$ + 3·5 ml. 10 p.c. $CuSO_4.5H_2O$. The unknown is diluted with 1 p.c. hydrochloric acid to match the standard solution in uniform bore test tubes in a comparator, using daylight for illumination or is compared with various dilutions of the standard. A better colour match is obtained without the urine blank, as the cobaltous sulphate solution has a somewhat brownish tint; no appreciable errors are caused by this except with unusually dark urines. By comparison with results obtained with the spectrophotometric method, it was established that the cobaltous sulphate-copper sulphate standard is equivalent to a concentration of 3·4 μg. diazotisable amine per ml. in the final solution, i.e. to 17 μg. per ml. in the acidified urine. Amounts found by this method were in close agreement with those obtained by spectrophotometric measurement.

*Method Used in Some Earlier Experiments.*

Before we had synthesized N–(1–naphthyl) ethylene diamine and obtained ammonium sulphamate, a few of the earlier animal experiments were carried out by the following method:

The total volume (or half of it on the first day after T.N.T. dosing) of the daily urine of rats was used. To each ml. of acid-treated urine 0·3 ml. of 0·1 p.c. nitrite was added, followed after 3 minutes by 0·3 ml. of 5 p.c. urea and after further 2 minutes by 0·3 ml. of 1 p.c. dimethyl–α–naphthylamine in alcohol. After 5 minutes standing conc. sodium acetate solution was added dropwise, until the solution turned yellow and a permanent cloudiness of the naphthylamine appeared. After 15 minutes standing the dye was extracted three times, each with 5 ml. ether in a

50 ml. separation funnel. The collected ether extracts were washed once with 10 ml. of distilled water, and the azo-dye extracted as hydrochloride by repeated shakings with 20 p.c. hydrochloric acid, using 2 ml. for the first and 1 ml. for subsequent extractions, until no further dye was extracted. The extracts were collected in small measuring cylinders, allowed to stand open until the solution became clear by evaporation of dissolved ether and determined spectrophotometrically as described above. If $\epsilon$ was the extinction coefficient in 1 cm. layer, V the volume of original urine, f the fraction of acidified urine used for extraction (1 or 0·5), v the volume of the acid extract, and b the daily excretion of diazotisable amine by the rat before dosing, the daily excretion of T.N.T. as diazotisable amine was measured as $\left(\frac{1{,}000\,\epsilon\,V\,v}{140\,f} - b\right)\mu g$. A comparison with the spectrophotometric method described above showed that much lower values (only about one-third as high) were obtained by this method, but that the results were parallel.

The losses were due (1) to incomplete coupling with dimethyl–α–naphthylamine, (2) to partial destruction of the diazonium compound by excess nitrite during standing and also after addition of urea, since urea does not destroy the nitrite completely, (3) to incomplete extraction of the dye by ether. This extraction and the re-extraction by hydrochloric acid were necessary, as nitrite, urea and dimethyl–α–naphthylamine (even when specially purified) produced a certain amount of yellow dye absorbing at 530 mμ; and this dye was not extracted from ether by HCl.

The formation of this dye can be prevented by the use of sulphamate instead of urea, allowing estimation directly in the urines as with naphthyl ethylene diamine. Even so the extinction developed with dimethyl–α–naphthylamine was only 30 to 70 p.c. of that developed with naphthyl ethylene diamine; by buffering to pH 4, followed by reacidification, it could be increased to 70–90 p.c. The following experiments carried out with 2.4–diamino–6–nitrotoluene, show that the lower extinction is not a property of the azo-dye, but is due to incomplete coupling with dimethyl naphthylamine. The molar extinction coefficient of the dye derived from the diamine was 32,200 if the coupling was carried out with naphthyl ethylene diamine, but only 11,900 after coupling with dimethyl–α–naphthylamine in acid solution and 18,000 after coupling in buffered solution, followed by re-acidification. The crystalline azo-dye with dimethyl–α–naphthylamine, however, purified by chromatographic absorption, had a molar extinction coefficient of 30,900, very similar to that of the naphthyl ethylene diamine dye.

*Extinction Coefficients of the Azo-dyes prepared by coupling Diazotised T.N.T. Reduction Products with Naphthyl Ethylene Diamine.*

Table 3 gives the molar extinction coefficients of the azo-dyes prepared from various T.N.T. reduction products added to the urines and diazotised and coupled in the manner used for the estimation of diazotisable amines in urine (cf. also the third paper of this series). No significant difference was observed between rats' and human urines, and approximately the same values were obtained, whether the amino compound was added to urine or to water, subtracting of course in the first case the blank due to presence of diazotisable amino compounds in the urines. The values for extinctions in water are given in the second column.

TABLE 3.

*Molar extinction coefficients of azo-dye hydrochlorides from T.N.T. reduction products at λ = 530 mμ.*

| | In urine. | In water. |
|---|---|---|
| 4–amino–2.6–dinitrotoluene | 34,700 | 36,300 |
| Cpd. m.p. 129°* | 21,800 | 23,100 |
| 2.4–diamino–6–nitrotoluene | 32,200 | 32,200 |
| 5–nitro–*m*–phenylene diamine | 38,700 | 40,900 |
| 2–nitro–4–aminophenol | — | 36,700 |

* This was isolated from human T.N.T. urine and later found to consist of a mixture of 6–amino–2.4–dinitrotoluene with 4–amino–2.6–dinitrotoluene. In stronger HCl and 50 p.c. alcohol $\epsilon$ mol = 31,000.

According to the analyses described in detail in the third paper of this series the diazotisable amines in rats' urine comprise about 30 p.c. of dinitrotoluidines, 55 p.c. of diamino–nitro compounds and 15 p.c. of aminonitrocresols, while those of human urines comprise 75 p.c. of dinitrotoluidines, 15 p.c. of diaminonitro compounds and 10 p.c. of aminonitrocresols. 2.4–diamino–6–nitrotoluene and 5–nitro–*m*–phenylene diamine occurred in about equal proportions in the rats' urine; the same proportion is assumed for human urines, although no separation was effected; since the percentage of diaminonitro compounds in human urine is small, the results would not be greatly affected, if the whole of this fraction were diaminonitrotoluene. In human urine the 4–amino compound and the substance m.p. 129° occurred in about equal proportion; since the latter still

includes some 4–amino compound, this agrees with the ratio of 4–amino to 6–amino compound as 7 : 3 which Channon *et al.* (1944) observed in rabbits' urine. This ratio is therefore, also assumed for rats' urine, although only 4–amino compound was isolated; here again the percentage of monoamino compounds is small. No aminonitrocresols have been studied; it is assumed that their extinctions would not differ greatly from those of 2–nitro–4–aminophenol. On this basis an average molar extinction coefficient of 33,300 for the diazotisable amino compounds of rats' urine and 30,200 for those of human urine is calculated. Both agree well with that used in in the analysis ($31{,}800 = \epsilon_{sp}^{T.N.T.} \cdot 140$).

*Acid Treatment of Urines.*

Urines of T.N.T.-dosed rats, when not heated with hydrochloric acid, gave only 50–90 p.c. of the diazotisable amines found after hydrolysis. Channon *et al.* (1944) have observed the same with rabbits' urines. They have ascribed the phenomenon to the conversion of 4–hydroxylamino-dinitrotoluene into azoxy and amino compound during the heating with acid. Pure 4–hydroxylamino–2.6–dinitrotoluene (cf. the third paper of this series) gives indeed an almost negligible diazo reaction and is converted into the 4–amino compound by acids. Nevertheless this explanation is not applicable to rats' urine, since no trace of hydroxylamino compounds can be discovered in them. Some of the amino groups must therefore be substituted, probably by acetyl groups.

## SUMMARY.

Spectrophotometric and colorimetric methods are described for the estimation of diazotisable amines in the urine of men and rats, which are formed from T.N.T. by reduction in vivo. These methods are based on the properties of the amino compounds isolated from the urines of rats and men.

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# METABOLISM OF AROMATIC NITRO COMPOUNDS

## 2. EXCRETION OF DIAZOTISABLE AMINES IN THE URINE AFTER INTAKE OF T.N.T. AND OF REDUCTION PRODUCTS OF T.N.T.

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While several workers (literature quoted in the first paper of this series) have shown that the ingestion of T.N.T. is followed by an increase in the excretion of diazotisable amines in the urine, so far no extensive quantitative studies have been published which show to what extent the excretion of diazotisable amine in the urine is proportional to the intake of T.N.T. and what proportion of T.N.T. is excreted in this form. In this paper we report on a series of experiments carried out mainly with rats, and describe also a few experiments with human volunteers.

In the second part a number of experiments is described in which the excretion of diazotisable amines was studied after feeding to rats various reduction products of T.N.T. The 4-azoxy compound has been assumed by Dale (1921) to be a metabolic reduction product of T.N.T., while 4–hydroxylamino–2.6-dinitrotoluene has been found in rabbits and men, 4-amino–2.6-dinitrotoluene in men, rabbits and rats and 2.6 diamino–4-nitrotoluene in rats (*cf.* the third paper of this series and Channon *et al.*, 1944).

### EXPERIMENTAL.

*Collection of Urine from Rats.*

Adult white rats of Wistar strain were fed a diet consisting of 67 p.c. wheat meal, 8·5 p.c. meat meal, 12 p.c. bone meal, 10 p.c. lactogen and 2·5 p.c. dried yeast. At least one week before commencement of the experiment the rat was isolated in a metabolism cage, consisting of a cylindrical vessel of wire mesh with wire mesh bottom. It contained a small side opening for the glass tubing from the inverted water flask which was fixed outside. The whole cage stood on a funnel through which urine and faeces fell on to a glass bulb topping a small Erlenmeyer flask, the latter being placed in a metal container. The faeces rolled off this bulb into the metal container while the urine was collected in the flask.

The animals were supplied with water *ad libitum* from the flask, but were fed outside the cage for two hours each day. In the experiments in which T.N.T. was applied in the food, the amount of food consumed was weighed. The rats soon accustomed themselves to this procedure of feeding; they lost weight during the first few days, but soon began to eat and drink vigorously and to gain weight.

The urine was collected each day and its volume measured. There was usually no loss of urine during feeding time, during which no water was given. If urinary excretion during feeding was noticed, the urine was collected with the day's specimen. Funnel and glass bulb were washed with a small amount of water which was added to the urine; and the urine was filtered.

The spectrophotometric method described in the preceding paper was used for the estimation of diazotisable amines with the exception of a few experiments with repeated single doses, which were carried out by the method described on p. 3 of the preceding paper.

*Single Doses of T.N.T.*

In these experiments the rats were dosed with a fine suspension of T.N.T. in a few ml. of milk which were pipetted directly into the oesophagus. The T.N.T. was first grade T.N.T. recrystallized from alcohol, m.p. 81° C. All the rats used in these experiments were males. The results are summarized in Table 1. The untreated animals excreted betwen 100 and 280 $\mu$g. diazotisable amine per day as average of four days, while the single daily excretions varied from 40 to 360 $\mu$g. After doses varying from 5 to 40 mg. of T.N.T. (25–250 mg. per kg.) there was a large increase of the excretion of diazotisable amine on the day following the dose, decreasing gradually, more rapidly after a small dose than after a large one. Even after 7 days the

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excretion was still somewhat above that before dosage. These results indicate storage of T.N.T. in a form still available for gradual excretion as diazotisable amine. In spite of considerable variations from individual to individual, both the response on the first day and the weekly total excretion of diazotisable amine in excess of normal are roughly proportional to the dose, except with the highest dose, where the excretion was more gradual and lower in percentage. Between 10 and 20 p.c. of the ingested T.N.T. was excreted as diazotisable amine; these excretions have to be considered as minimum figures, since the excretion had not yet returned to normal.

Rat 31, which excreted an exceptionally large amount of diazotisable amines, died three days after a moderate dose. This suggested that a particularly toxic substance might occur among the reduction products of T.N.T. and individual susceptibility be explained by its formation. Later on, however, we observed early deaths of rats after single doses of T.N.T. with normal and even subnormal excretion of diazotisable amines, and none of the reduction products of T.N.T. isolated from the urines of rats proved to be very toxic. The death of rat 31 may not have been due to T.N.T.

TABLE 1.

*Diazotisable amines after a single dose of T.N.T. per os.*

| Rat No. | 35 | 34 | 31 | 36 | 32 |
|---|---|---|---|---|---|
| Weight g. | 190 | 209 | 261 | 220 | 161 |
| Normal excretion μg. per day | 150 | 105 | 205 | 155 | 275 |
| T.N.T. dose, mg. | 5 | 10 | 20 | 20 | 40 |
| Excretion μg. per day | | | | | |
| 1st day | 560 | 785 | 2,910 | 1,710 | 1,080 |
| 2nd day | 45 | 245 | 1,100 | 360 | 775 |
| 3rd day | 340 | 170 | 390 | 205 | 905 |
| 4th day | 325 | 270 | died | 375 | 670 |
| 5th day | 115 | 200 | — | 135 | 365 |
| 6th day | 460 | 245 | — | 460 | 210 |
| 7th day | 275 | — | — | 160 | 450 |
| Total excretion above normal mg. | 1·06 | 1·27 | 3·79 | 2·01 | 2·53 |
| P.c. of this on first day | 39 | 53 | 74 | 62 | 32 |
| Recovery of T.N.T. p.c. | 21 | 13 | 19 | 10 | 6·5 |

Our rats survived doses as high as 1 gm. T.N.T. per kg., given suspended in warm olive oil. The median lethal dose was certainly higher than the 0·7 g./kg. assumed as probable by Wyon (1921). It depends perhaps on the presence of protective factors in the diet.

*Single Doses of T.N.T. Repeated.*

In this series T.N.T. was given per os dissolved in warm maize oil. The animals were acutely ill soon after dosing, but most of them survived even repeated high doses. It has been reported that the toxic effect of T.N.T. is increased by the ingestion of fats, and the amount of oil required for dissolving T.N.T. was rather large (0·5–2 ml.) and may in itself have caused acidosis.

Table 2 gives the results. A strict comparison with the results of the first series is not possible, since the earlier urea-dimethyl naphthylamine method with ether extraction was used, multiplying the results by 3, the factor obtained by several determinations on one and the same solution by the two methods. If it is taken into account that higher doses decrease the recovery and that the doses applied in this series were higher than in the first series, it is seen that the recoveries are of about the same magnitude.

TABLE 2.

*Diazotisable amine excretion after repeated single doses of T.N.T. per os.*

| No. | Rat weight. | Normal excretion mg. per day. | First dose mg. T.N.T. | Excretion mg. | | Recovery p.c. | Second dose mg. T.N.T. | Excretion mg. | | Recovery p.c. | Third dose mg. T.N.T. | Excretion mg. | | Recovery p.c. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | In first 3 days. | Average per day in following weeks. | | | In first 3 days. | Average per day in following weeks. | | | In first 3 days. | Average per day in following weeks. | |
| 22 | 350 gm. male | 0·10 | 25 | 1·26 | 0·20, 0·19, 0·18 | 12 | 47 | 3·61 | 0·43, 0·22 | 13 | — | — | — | |
| 23 | 270 gm. female | 0·10 | 25 | 1·56 | 0·23, 0·21, 0·23 | 16 | 47 | 2·42 | 0·36, 0·30 | 11 | — | — | — | |
| 15 | 190 gm. male | 0·06 | 95 | 4·29 | 0·14, 0·19, 0·09 | 6 | 47 | 3·14 | 0·18, 0·11 | 9 | 47 | 3·10 | 0·12, 0·17 | 7 |
| 14 | 135 gm. female | 0·13 | 34 | 2·08 | 0·20, 0·12, — | 7 | 34 | 2·50 | 0·13, 0·13 | 6 | 47 | 3·87 | 0·10, 0·22 | 9 |

After the second and third dose the recovery was the same as after the first. Small amounts of diazotisable amine in excess of the excretion before dosing still continued to be excreted for about 3 weeks after the dose, and the level of this small slow excretion was slightly increased by each dose. This again indicates the storage of T.N.T. in a form available only for slow urinary excretion as diazotisable amine, but no direct experimental evidence for this storage could be found (cf. below).

*Continuous Dosing of Rats per os.*

In these experiments T.N.T. was mixed with the food as a fine powder and the intake was measured by weighing the food consumed each day. By varying the percentage of T.N.T. in the food the dose could be adjusted to the desired level, but slight variations owing to variations in food intake could not always be avoided. In order to accustom the animals to the food, smaller doses were given at the beginning. On a level of dosing of 3 mg. T.N.T. per gm. food only 2 of 12 rats survived 13 days. On Sundays the animals received no food so that the urine collected on Monday represented a two days specimen following the dose given on Saturday. All the animals were males.

TABLE 3.

*Diazotisable amine excretion on continuous dosing with T.N.T. admixed to food.*

| No. | Rat weight. gm. | Duration of dosing, weeks. | Level of dosing, mg. T.N.T. per gm. food. | Total T.N.T. consumed, mg. | Average percentage of of weekly intake recovered. | Weight at death, gm. |
|---|---|---|---|---|---|---|
| 46 | 167 | 17 | 11 weeks 0·13–0·45<br>6 weeks 1·0 | 502 | 13·4 | 119 |
| 53 | 163 | 6 | Rising from 0·4 to 1·4 | 168 | 10·7 | 97 |
| 54 | 216 | 15 | 2 weeks 0·5<br>2 weeks 1·0<br>11 weeks 1·5 | 1,060 | 10·0 | 135 |
| 45 | 122 | 33 | 10 weeks 0·13–0·45<br>23 weeks 1·0 | 1,570 | 11·3 | 208 survives |

The average weekly recoveries in the experiments with continuous dosage were somewhat lower than the recoveries after a single dose. This is probably due to the delay in excretion which caused effects noticeable in the weekly percentage of recovery. The recoveries for the first week or fortnight at the beginning of the experiment or immediately after a much lower preceding dose were lower than those afterwards, and exceptionally high recoveries were observed when a smaller dose was given after a larger one. We also observed a distinct rhythmic variation of daily recoveries according to week days. The smallest average recovery was found in the urines excreted on Mondays, one day after the omission of the dose on Sundays, and it gradually rose towards the end of the week. The probable error of the single day recoveries was 30 p.c., that of the weekly recoveries 17 p.c.

There appears to be a correlation between the volume of the urine excreted and the percentage of T.N.T. recovery as shown by the figures in Table 4.

TABLE 4.

*Relation of recovery of T.N.T. as diazotisable amine to volume of urine.*

| Volume of urine c.c. | P.c. recovery. | As average of days. |
|---|---|---|
| $< 5$ | 7·9 | 47 |
| 5–10 | 11·8 | 57 |
| 10–15 | 12·8 | 52 |
| 15–20 | 14·7 | 15 |
| $> 20$ | 14·95 | 10 |

This effect may however have been caused by occasional loss of urine with the faeces or during feeding. Channon *et al.* (1944) have observed larger excretion of diazotisable amines by the rabbit on wet feeding than on dry feeding, but no such difference was found in rats fed 4–amino–2.6–dinitrotoluene (cf. Table 6). The higher values of about 15 p.c. recovery are therefore more likely to be correct.

The animals 53 and 54 lost weight, at first slowly, later rapidly, although they ate their normal amounts of food until shortly before death. Rats 45 and 46 gained weight for the first nine weeks, remained stable for several weeks and then lost weight rapidly. While rat 46 died after 17 weeks, rat 45, a younger rat, began to increase in weight again from the 20th to the 26th week, and gained rapidly during the Christmas and New Year holidays when it received T.N.T.-free food for eight out of twenty-one days. It then weighed 227 gm., 100 gm. more than at the beginning of the experiment. After this it began slowly to lose weight again, but survived 5 more weeks on a dose of about 1 mg. per gm. food.

It is particularly important that the percentage recovery remained unchanged right up to the death of the animals (average of last weekly recoveries before death in 3 animals, 13·6 p.c.). The Webster test becomes negative in the later stages of cases of human T.N.T. poisoning, and therefore useless for diagnosis. The determinations of diazotisable amines in the urine will still supply a quantitative estimate of T.N.T. intake, or excretion of stored T.N.T. products.

*Continuous Dosing of Rats through the Skin.*

In these experiments a solution of T.N.T. in lanolin (100 mg. in 4 gm.) was rubbed into a shaved area of abdominal skin. The results of Table 5 show that the average weekly recoveries are of the same size as with T.N.T. applied per os.

TABLE 5.

*Diazotisable amine excretion in continuous dosing with T.N.T. through the skin.*

| No. | Rat weight, sex. | Duration of dosing, weeks. | Level of dosing. | Average weekly recovery, p.c. |
|---|---|---|---|---|
| 51 | 167 gm. male | 5 | a | 8·0 |
| | | 5 | b | 11·2 |
| 52 | 198 gm. female | 5 | a | 7·8 |
| | | 6 | b | 13·6 |
| 44 | 172 gm. female | 4 | a | 16·5 |

a 2·5 mg. T.N.T. in 0·1 gm. lanolin 3 times a week on an area of 3 cm².
b 5·0 mg. T.N.T. in 0·2 gm. lanolin 3 times a week on an area of 6 cm².

All the animals died after the time given in Table 5, but it is doubtful whether their deaths were caused by T.N.T. The weight remained steady until the last week, when a sharp drop occurred.

The fur of the animals acquired a peculiar brownish-pink colour, at first mainly on the ventral surface and tail, later all over the body. The pigment could not be removed by extraction with ether or other organic solvents except pyridine. Pyridine gave a red extract which slowly turned orange on standing. 2.4.6–trinitrobenzyl alcohol (cf. Channon *et al.*, 1944) behaves similarly, and produces a red stain on human skin. We noted appearance of pink pigment near the penis and scrotum or vagina of the rats dosed with T.N.T. after a time varying from 30 to 400 minutes, according to the dose of T.N.T. applied. According to Channon *et al.* the pink pigment observed in freshly voided rats' urine is trinitrobenzyl alcohol or a reduction product of it. We found that by adding trinitrobenzyl alcohol to fresh rats' urine the pink colour is produced and that it becomes very strong if dilute ammonia is added. The pink colour is thus probably derived from trinitrobenzyl alcohol itself. The rat does not reduce trinitrobenzyl alcohol to diazotisable amine.[2] No increase of the excretion of the latter in urine was observed if trinitrobenzyl alcohol was ingested with the food. The widespread coloration of the fur observed with skin-dosed animals was never observed in rats fed T.N.T. It therefore appears that in these animals trinitrobenzyl alcohol is produced in the skin itself. The fact that the colour was also found in parts of the skin not easily reached by the paws of the animal indicates, however, that it is not distributed superficially, but passes through the circulation and is deposited in skin and hair.

Standing of the defatted pink hairs in 5 p.c. HCl for 48 hours gave an extract containing diazotisable amines, but so did normal white hair subjected to the same treatment. The ether extract of the pink hairs did not contain diazotisable amines.

*Continuous Dosing of Rats per os. with Reduction Products of T.N.T.*

*2.6.2'.6'–tetranitro–4.4'–azoxy–toluene* was obtained as by-product in the reduction of T.N.T. to 4–amino–2.6–dinitrotoluene with $H_2S$ in ammoniacal dioxane (cf. below). The mother liquors were concentrated, when white crystals separated out which were sparingly soluble in water,

[2] Later it was found that after an induction period of ten days rats continually dosed with trinitrobenzyl alcohol in the food excrete diazotisable amines in the urine, the recovery remaining, however, low (5 p.c.).

acetic acid and alcohol. They were extracted by boiling with 40 p.c. acetic acid in order to remove amino compounds, and recrystallized from dioxane by addition of water and then from benzene. White crystals, m.p. 213° (uncorr.). The substance was also obtained by heating the 4–hydroxylamino compound for 2 hours at 100° C. with conc. HCl.

*4–hydroxylamino–2.6–dinitrotoluene* was produced by the method of Elvove (1919). The m.p. of the product (135–136° C.) could not be raised by further recrystallization. It was found that this preparation still contained an admixture of 4–amino–2.6–dinitrotoluene. If it was dissolved in ether, 20 p.c. hydrochloric acid extracted a substance which gave a strong red colour after diazotisation and coupling with naphthyl ethylene diamine. This was identified as 4–amino–2.6–dinitrotoluene (m.p. 171° C. and mixed m.p.). On further extractions with 20 p.c. HCl no further diazotisable amine was removed. The whole product of m.p. 135°-136° C. was therefore dissolved in peroxide-free ether, the ether solution washed once with an equal volume of 20 p.c. HCl and then twice with distilled water, dried over anhydrous sodium sulphate and evaporated. The residue was taken up in a little alcohol, leaving a trace of azoxy compound undissolved. On concentration of the solution the hydroxylamino compound crystallized out and was washed with a little cold benzene. Well-formed yellow prismatic needles of square or hexagonal cross section with pyramidal ends, frequently consisting of many small facets; probably monoclinic; m.p. (corr.) 150°–151° C. Readily soluble in alcohol, soluble in ether and warm benzene, sparingly in cold benzene, very slightly soluble in water.

Analysis. Found: C, 39·72, 39·53; H, 3·26, 3·50; N, 19·8, 20·0. Calc. for $C_7H_7O_5N_3$: C, 39·42; H, 3·31; N, 19·7.

The alcoholic solution reduces ammoniacal silver nitrate rapidly in the cold and Benedict's solution on boiling. On boiling for two hours with conc. HCl it is converted into a mixture of the 4–amino and the 4–azoxy compound, but is much more stable than the impure product, alcoholic solutions of which deposit the azoxy compound on standing for a few minutes.

On diazotisation in 2 p.c. hydrochloric acid, containing some alcohol, and coupling with naphthyl ethylene diamine, it gives a weak brown colour, which gradually changes to a very weak purple. Somewhat more purple colour is developed if 10 or 20 p.c. hydrochloric acid is used, probably due to a conversion of a small part into the 4–amino compound. If the crystals are dissolved in ether, underlayered with 10 p.c. HCl and the diazo reagents added in quick succession with very vigorous shaking after each addition in order to destroy by sulphamate the nitrous acid dissolved in the ether, no colour results, while the 4–amino compound gives an intense purple colour. The preparation, therefore, no longer contains 4–amino compound and does not give the diazo reaction itself.

In the Webster test, the hydroxylamino compound gives a brownish purple reaction, more purple when a larger, more brown when a smaller amount of alcoholic sodium hydroxide is added. The Webster test is carried out by us in the following modification: 2–3 ml. of the ethereal solution (if wet, dried with anhydrous $Na_2SO_4$) are diluted to 4 times its volume with alcohol, and 2 drops of conc. alcoholic NaOH solution are added.

The N–(3.5–dinitro–4–methyl phenyl) isobenzaldoxime prepared from our product according to Channon *et al.* (1944) had a m.p. of 224° C., 11 degrees higher than reported by these authors.

*4–amino–2.6–dinitrotoluene* was prepared by Mr. Legge by dissolving 1 gm. T.N.T. in 20 ml. dioxane adding 0·2 ammonium hydroxide (s.g. 0·880). $H_2S$ was bubbled in with cooling The sulphur was filtered off, 5 ml. of alcohol and 1 ml. acetic acid were added to the filtrate and then, slowly with shaking, water until crystals separated out. Recrystallization from 40 p.c. acetic acid gave a 33 p.c. yield of 4–amino–2.6–dinitrotoluene, m.p. 166° C. (corr.). By recrystallization from chloroform with the addition of light petroleum the m.p. was raised to 175° C. (corr.). The acetyl compound was obtained by acetylation with acetic anhydride in pyridine as straight prismatic needles, m.p. 222° C. (uncorr.). A larger sample was kindly supplied by the Munitions Supply Laboratories; it had been prepared by $H_2S$ reduction in ammoniacal alcohol and melted at 170° C.

TABLE 6.

| No. | Rat weight, gm. | Compound fed. | Duration of dosing days. | Level of dosing, mg. per gm. food. | Total dose as mg. T.N.T. | Overall recovery, p.c. |
|---|---|---|---|---|---|---|
| 62 | 240 | 4–azoxy–2.6–dinitrotoluene | 30 | 1·0 | 170 | 0·6 |
| 63 | 236 | 4–hydroxylamino–2.6–dinitrotoluene | 6 | 1·0 | 62 | 13·2 |
| | | | 13 | 1·5 | 262 | 14·8 |
| 88 | 285 | 4–amino–2.6–dinitrotoluene | 10 dry food | 1·0 | 105 | 15·5 |
| | | | 10 wet food | 1·0 | 172 | 15·2 |
| 58 | 284 | 2.4–diamino–6–nitrotoluene | 12 | 0·7 | 185 | 17·5 |
| | | | 7 | 1·0 | 74 | 18·7 |
| | | | 27 | 1·5 | 378 | 19·2 |

*2.4–diamino–6–nitrotoluene* was prepared according to Ruggli (1936). After recrystallization from chloroform by addition of light petroleum the m.p. was 138° C. (corr.). The acetyl derivative was prepared by acetylation with acetic anhydride in pyridine. Recrystallization from water gave almost colourless fine needles, m.p. 270° C. (uncorr.).

In the experiments described in Table 6 these compounds were fed to rats admixed to the food as fine powder. The recoveries are calculated in p.c. of T.N.T. corresponding to the dose given of the particular compound, using $\epsilon_{sp.}$ T.N.T. = 140 to calculate the yields of diazotisable amines except in the experiment with the diamino nitro compound, in which $\epsilon_{sp.}$ T.N.T. = 155 was used (cf. the first paper of this series). All the animals were males.

Very little diazotisable amine was formed from the 4–azoxy compound which makes it unlikely that this substance is an intermediate in the formation of the amino compounds.

The 4–hydroxylamino compound, however, yielded as much diazotisable amine as T.N.T., which makes the hydroxylamino compound the most likely first reduction product of T.N.T. In agreement with this is the occurrence of the compound in human and rabbit T.N.T. urines (Channon *et al.*, 1944), which we also observed (cf. the third paper of this series). The rat fed with 4–hydroxylamino compound died after 18 days, death having probably been caused by exposure to cold; in spite of dosing with comparatively high doses (1·5 mg. per gm. food) the weight of the animal did not decrease. The compound is therefore not as toxic to the rat as to the other animals (cf. Wyon, 1921, p. 47). This is explained by a stronger reduction in the rat indicated by the formation of diamino nitro compounds. We have never observed the purple Webster test characteristic of the hydroxylamino compound in the urine of rats dosed with T.N.T., and not even in those dosed with hydroxylamino compound itself. A blood catalase estimation on T.N.T.-dosed rats did not indicate activity lower than that of normal rats; a reduced activity would be expected were the hydroxylamino compound present in the blood.

4–amino–2.6–dinitrotoluene gave about the same percentage recovery as T.N.T. or the 4–hydroxylamino compound, and this was not influenced by the use of dry or wet food, although the dry-fed rat excreted 114 ml. of urine while the wet-fed rat excreted 195 ml.

2.4–diamino–6–nitrotoluene gave distinctly high recoveries, although the higher specific extinction of the 5–nitro–*m*–phenylene diamine formed from the toluene compound had been taken into account in the calculation. Occasionally they reached 30–35 p.c., but on the average they were 20 p.c. The greater part, therefore, disappears, and the loss of T.N.T. in the rat must be caused to a greater extent by further decomposition of the diamino compounds, than during the reduction to the diamino stage. The replacement of amino by hydroxy groups, perhaps after reduction to the unstable triamino stage may be the mechanism of this destruction.

*Excretion of Diazotisable Amines in the Faeces.*

Normal rats' faeces contained 250–450 $\mu$g. diazotisable amine per day. Neither T.N.T. nor 2.4–diamino–6–nitrotoluene caused significant increases. A rat fed T.N.T. and excreting 2 mg. per day as diazotisable amines in the urine, still excreted only 250 $\mu$g. in the faeces. A rat fed 2.4–diamino–6–nitrotoluene and excreting 2·6 mg. per day in the urine, excreted in one experiment 600 $\mu$g., in another only 150 $\mu$g. in the faeces.

*Attempts to Discover Stored T.N.T. or Aromatic Amino Compounds in the Organs.*

Rat 45 had ingested 1·6 gm. of T.N.T. in 33 weeks, and excreted as diazotisable amine only 0·18 gm. It received T.N.T. in its food until one day before it was killed. The investigation for unaltered T.N.T. was carried out by grinding the tissues with sand and acetone, filtering and adding 1–2 drops of 10 p.c. alcoholic potassium hydroxide. A very faint red colour, indicating a trace of T.N.T., was obtained with muscle extract, none with the extracts of organs, while a trace of T.N.T. added produced a deep brown-red to purple colour. For diazotisable amines the organs were extracted three times by grinding with sand under 10 p.c. trichloracetic acid. Only traces of diazotisable compounds were found in kidney, peritoneum, small intestine and muscle, while the liver contained somewhat more (0·25 mg. in the whole liver).

A rat which had received a single dose of 150 mg. 2.4–diamino–6–nitrotoluene was killed 8 days after dosing. The organs contained only very small amounts of diazotisable amines. The whole liver contained 38 $\mu$g., the kidney 24 $\mu$g., the lungs 13 $\mu$g., peritoneum 9 $\mu$g., heart 7 $\mu$g.; none was found in muscle, spleen and intestine.

There is thus little evidence for prolonged storage of large amounts of T.N.T. unaltered or in the form of amino compounds. The large difference between T.N.T. absorbed and excreted as diazotisable amines can either be due to storage in an unknown form, or, more likely to destruction of the reduction products in the body.

*Single Doses of T.N.T. given per os to Human Volunteers.*

The normal daily excretion of diazotisable amine in human urines varied between 7 and 13 mg. per day. 24-hour specimens of the urine of 5 volunteers were collected for one day before dosing and single doses of 10–30 mg. T.N.T. were given in milk. The urine was collected for 24 hours after dosing, in two cases of higher dosing for 48 hours. While after a dose of 20 mg. the second day excretion had returned to normal, it was still increased after 30 mg.

TABLE 7.

*Diazotisable amine excretion in human urine after single doses per os.*

| | Normal excretion. mg. per day. | Dose, mg. T.N.T. | Excretion after dose, mg. per day. 1st day. | 2nd day. | Recovery, p.c. |
|---|---|---|---|---|---|
| S | 10·5 | 10 | 14·1 | — | 36 |
| Sp | 12·9 | 10 | 17·4 | — | 45 |
| H | 10·1 | 20 | 14·5 | — | 22 |
| L | 11·3 | 20 | 23·8 | 9·5 | 54 |
| C | 7·8 | 30 | 19·2 | 14·2 | 60 |

In each case the dose was followed by a distinct increase of diazotisable amine. As is to be expected with such small doses, the recoveries varied considerably, but are evidently higher (average 44 p.c.) than in the rat. The same appears to hold for the rabbit (Channon *et al.*, 1944). Since in both men and rabbits the main products of T.N.T. reduction are hydroxylamino- and amino-dinitro compounds, while in the rat reduction proceeds further, mainly to the stage of diaminonitro compounds, these differences in recovery are probably due to the lesser stability of the more highly reduced amino compounds in the body.

Only four urines of munition workers were available; one contained a normal amount (6 μg. per ml.), another 17 μg., a third 148 μg. and the fourth 153 μg. per ml. From the last two were isolated the reduction products of T.N.T. described in the third paper of this series.

## SUMMARY.

The excretion of diazotisable aromatic amino compounds in the urine of rats and men is always roughly proportional to the intake of T.N.T., and remains constant in rats until the day of death. About 20 per cent. of a single dose of T.N.T. given per os to rats can be recovered in the urine of rats as diazotisable amine. Most of this is execreted on the first day, but smaller amounts continue to be excreted for several days afterwards. If the dose is repeated, the initial response after the later doses is the same, but the level of slow gradual excretion of diazotisable compounds is increased after each dose. On continuous dosing with T.N.T. 10–15 per cent. is excreted as diazotisable amines in the urine, and the experiments indicate again some storage. Nevertheless no direct evidence for such storage, either in the form of unaltered T.N.T. or as aromatic amino compounds, could be detected. Continuous dosing of T.N.T. through the skin causes the same average excretion of aromatic amines as ingestion. No diazotisable amine is execreted in the faeces.

Human volunteers excreted on the average 44 per cent. of small doses of ingested T.N.T. as aromatic amino compounds in the urine.

While the ingestion of 4-azoxy–2.6-dinitrotoluene by rats does not lead to an appreciable excretion of aromatic amines in the urine, 4-hydroxylamino–2.6-dinitrotoluene and 4-amino–2.6-dinitrotoluene cause excretion to the same extent as T.N.T., and 2.4-diamino–6-nitrotoluene to a slightly higher extent. The loss of T.N.T is therefore largely due to the destruction of its reduction products in the body.

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# METABOLISM OF AROMATIC NITRO COMPOUNDS

## 3. ISOLATION OF REDUCTION PRODUCTS OF 2.4.6–TRINITROTOLUENE FROM THE URINE OF RATS AND FROM HUMAN URINE.

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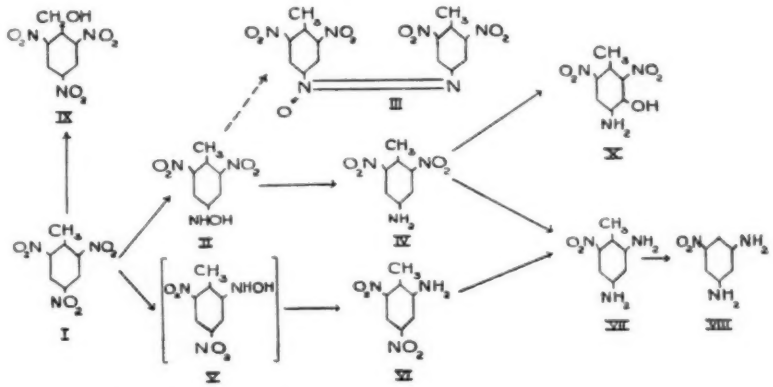
When our studies began, little was known about the nature of the substances into which T.N.T. is transformed in the organism. 2.2′–6.6′–tetranitro–4.4′–azoxytoluene (III in Table 1) had been isolated from the urines of munition workers, monkeys and rabbits (Moore, 1917; Webster, 1921; Dale, 1921). Moore assumed—as we know now correctly—that the azoxy compound was formed by oxidation of 4–hydroxylamino–2.6–dinitrotoluene (II) during the isolation, while Dale and Webster came to the conclusion that the azoxy compound itself was excreted. The latter authors also obtained a dinitrotoluidine, the identity of which they could not, however, establish.

Recently Channon *et al.* (1944) have published an extensive paper on the metabolic products obtained from T.N.T. From rabbits' urine they isolated 4-hydroxylamino–2.6–dinitrotoluene (II), 4–amino–2.6–dinitrotoluene (IV) and 2–amino–4.6–dinitrotoluene (VI).

TABLE 1.



Our investigations were carried out with rats and with the urine of two munition workers particularly rich in diazotisable amine. We describe a method by which the dinitrotoluidines (IV and VI), diaminonitro compounds (VII and VIII), aminonitrocresols (*e.g.* X) and hydroxylamino compounds (II, V) can be separated. The main products in human urine are the same as those found in rabbits' urine by Channon *et al.* (II, IV, and VI), *i.e.* dinitro-hydroxylamino and dinitro-amino-toluenes. In addition to these we found some diaminonitro compounds and aminonitrocresols. In rats' urine, the main products were, how-

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ever, diaminonitro compounds. In addition to 2.4–diamino–6–nitrotoluene (VII), 5-nitrophenylene diamine (VIII) was isolated from the urines of rats fed with T.N.T. as well as those fed with 2.4–diamino–6–nitrotoluene (VII). This is the first evidence that the methyl group of T.N.T. can be eliminated *in vivo*, and so far we know the only case of removal of the methyl group from the benzene nucleus in animal metabolism.

## EXPERIMENTAL.

*Separation of 4–amino–2.6–dinitrotoluene from 2.4–diamino–6–nitrotoluene.*

5 mg. of 4–amino–2.6–dinitrotoluene were dissolved in 50 ml. of ether and extracted repeatedly with 1 p.c. hydrochloric acid, 4 ml. being used for each extraction. The contents of the amine in the acid extracts was determined by diazotisation, coupling and spectrophotometric estimation of the azo-dye as described for the estimation of diazotisable amines in urine in the first paper of this series. It was found that one extraction with 1 p.c. hydrochloric acid removed 0·2 p.c. of the total amount of substance dissolved in ether, while three extractions removed 0·4 p.c.

The same experiment was carried out with 2.4–diamino–6–nitrotoluene. The first HCl extract contained 90 p.c. of this amine; three extractions removed 99·5 p.c.

5–nitro phenylene diamine (cf. below) is still more readily extracted by 1 p.c. hydrochloric acid than the nitrotoluylene diamine, while 2–amino–4.6–dinitrotoluene is still less basic than the 4–amino compound. The method gives thus a highly effective separation of the dinitrotoluidines from the diaminonitro compounds.

*Recovery of 4–amino–2.6–dinitrotoluene and 2.4–diamino–6–nitrotoluene added to urine.*

For the study of the absorption of a mixture of substances it is convenient to introduce the conception of "total absorption" ($\Sigma E$). In the solution of a single substance we have $\epsilon = \epsilon_{sp.} C = \epsilon_{sp.} g/v$, $\epsilon$ being the extinction coefficient, $\epsilon_{sp.}$ the specific extinction coefficient, g the amount of absorbing substance in mg. and v the volume of the solution in ml. The total absorption E is then defined as $\epsilon v = \epsilon_{sp.} g$. In a mixture of several absorbing substances, the law holds: $\Sigma E = e\,v = \epsilon_{sp.1}\, g_1 + \epsilon_{sp.2} g_2 + \epsilon_{sp.3} g_3 + \ldots$ each of the additive terms being either obtainable from the known specific coefficients and weight, or from measured extinction and volume.

To 480 ml. of acid-treated normal rats' urine we added 6·47 mg. of 4–amino–2.6–dinitrotoluene ($\epsilon_{sp.}^{530 m\mu} = 176$, E = 1,140) dissolved in alcohol, 6·17 mg. of 2.4–diamino–6–nitrotoluene ($\epsilon_{sp.}^{530 m\mu} = 193$, E = 1,190) dissolved in 1 p.c. hydrochloric acid and 7·12 mg. 4–hydroxylamino–2.6–dinitrotoluene (E = 100) dissolved in alcohol.

The acid urine was then subjected to continuous ether extraction (extract I), removing the 4–amino and 4–hydroxylamino compound, and the total extraction of the azo-dyes in the residual urine determined by diazotising and coupling a sample and determining the diazotisable amines spectrophotometrically as described in the first paper of this series. Decrease of total absorption by the first extraction: $\Delta \Sigma E = 990$. The same treatment of normal rats' urine without added amino compounds gave $\Sigma E = 140$ in the first extract, due to the presence of small amounts of ether-extractable diazotisable amines in normal rats' urine. $\Delta \Sigma E$ corrected = 850. Calculated for 4–amino and 4–hydroxylamino compound = 1,240. Recovery: 79 p.c.

The residual urines remaining after the first extraction were then made alkaline with solid sodium carbonate and subjected to a second continuous ether extraction (extract II). $\Delta \Sigma E$ of urine containing added amino compounds 1,180, or normal urine 80. $\Delta \Sigma E$ corrected: 1,100. Calculated for 2.4–diamino–6–nitrotoluene: 1,190. Recovery: 92 p.c.

A small part of ether extract I was retained for investigation of the hydroxylamino compound. The remainder was washed with N/5 sodium hydroxide and 4–amino–2.6–dinitrotoluene extracted with 20 p.c. hydrochloric acid; $\Sigma E = 810$. Recovery: 71 p.c.

Ether extract II was washed twice with a few ml. of sodium hydroxide and then extracted with 1 p.c. hydrochloric acid. $\Sigma E = 940$. Recovery of 2.4–diamino–6–nitrotoluene: 79 p.c. The diazotisable amines from normal urine are almost completely extracted by alkali, and the last two figures therefore need no correction. By washing ether solutions of 2.4–diamino–6–nitrotoluene with large amounts of water or dilute alkali, a considerable part of the amine can be removed.

A small part of extract I was extracted with 20 p.c. hydrochloric acid without previous extraction with alkali. The remaining ether solution contained largely unaltered 4–hydroxylamino–2.6–dinitrotoluene. The Webster test in alcohol-ether gave a purple colour with slightly bluish tinge, the latter being caused by some azoxy compound formed from the hydroxylamino compound during extraction.

A similar treatment of 1 l. normal rats' urine gave the results summarized in Table 2. The corrections for diazotisable amines mentioned above and in experiments described below are derived from these figures. The first ether extraction was of the urine acidified with HCl, the second of the urine made alkaline with $Na_2CO_3$ and a third of the residual urine acidified with acetic acid. Practically all diazotisable amine was extracted from the united extracts by N/5 NaOH, while sodium bicarbonate removed only the smaller part, probably mostly p–aminobenzoic acid. The remainder contains substances of unknown nature, different from p–aminobenzoic acid, which are being studied. They readily lose their ether-extractability on standing and appear to be unstable, but a stable azo-dye was obtained by coupling with dimethyl–α–naphthylamine.

TABLE 2.

*Diazotisable amines of normal rats' urine.*

| | Σ E |
|---|---|
| Total 1 l. urine | 1,790 |
| First ether extract | 290 |
| Second ether extract | 170 |
| Third ether extract | 250 |
| Residual urine | 1,020 |
| Not extracted by NaOH, extracted by 1 p.c. HCl | 40 |
| Not extracted by NaOH, extracted by 20 p.c. HCl | 60 |
| Extracted by $NaHCO_3$ | 240 |
| Not extracted by $NaHCO_3$, extracted by NaOH | 390 |

*Metabolic Products from the Urine of Rats dosed with T.N.T.*

815 ml. of urine were collected from a number of rats which received about 10 mg. T.N.T. daily in their food (1 mg. per 1 gm. food). The urine was acidified with one fiftieth of its volume of conc. HCl immediately after collection, and the collected urine was heated one hour on a boiling water bath. It was then subjected to the three extractions with ether. Table 3 shows that only somewhat more than 50 p.c. of the diazotisable amines could be extracted by ether.

TABLE 3.

*Diazotisable amines in the urine of rats dosed with T.N.T.*

| | Σ E. | Δ Σ E. |
|---|---|---|
| Total urine, 815 ml. | 10,000 | |
| Ether extract I | | 1,750 |
| NaOH extractable | 640 | |
| 1 p.c. HCl extractable | 50 | |
| 20 p.c. extractable | 1,210 | |
| Ether extract II | | 2,800 |
| NaOH extractable | 230 | |
| 1 p.c. HCl extractable | 2,360 | |
| 20 p.c. HCl extractable | 50 | |
| Ether extract III | | 800 |
| NaOH extractable | 450 | |
| 1 p.c. HCl extractable | 350 | |
| Residual urine | 4,670 | |
| Alkali extractable | 1,320 | |
| Monoaminodinitro | 1,260 | |
| Diaminonitro | 2,760 | |
| Corrected for Σ E from normal urine: | | |
| Aminonitrocresols | 890 | |
| Aminodinitrotoluenes | 1,210 | |
| Diaminonitro compounds | 2,730 | |

Each of the three ether extracts was concentrated to a small volume, extracted several times with small amounts of N/5 NaOH, then three times with a few ml. of 1 p.c. hydrochloric acid and finally repeatedly with 20 p.c. hydrochloric acid. In contradistinction to normal rats' urine, a

large part of the diazotisable amine derived from T.N.T. was not extractable by alkali, and the strongly basic diamino compounds, which form the major part of the second ether extract, prevailed. The alkali-extractable diazotisable amines were, however, also distinctly increased. The alkaline extracts were acidified at once and determined in the usual way. A part of the diazotisable amine found in the alkali extracts of the second and third ether extract may actually consist of nitrotoluylene diamine and nitrophenylene diamine, which are rather readily extracted by water from ether, but the fact that only a weak yellow colour appeared with nitrite indicates that this proportion cannot be great. Moreover, the alkali-extractable amino compounds were also increased in the first ether extract which does not contain diaminonitro compounds. Sodium bicarbonate extracted only a small part of these substances from ether. The remainder was evidently aminophenolic in character and consisted probably of aminonitrocresols. The first extract did not give any Webster test, and therefore, did not contain hydroxylamino compounds. The last three lines of Table 3 show Σ E of dinitrotoluidines, nitrotoluylene and nitrophenylene diamines and aminonitrocresols, corrected for Σ E of the corresponding fractions from normal rats' urine, a correction which mainly concerns the last. According to these figures, dinitrotoluidines cause 25 p.c., nitrotoluylene and nitrophenylene diamines 57 p.c. and aminonitrocresols 18 p.c. of the total absorption of ether-extractable diazotisable amines derived from T.N.T.

*Dinitrotoluidines.* Ether extract I was extracted by 20 p.c. hydrochloric acid. The ease with which a large part of the substance was extracted by the acid suggests that 4-amino-2.6-dinitrotoluene prevailed over 2-amino-4.6-dinitrotoluene (cf. below). The yellow solution was neutralized with solid sodium carbonate and the amine extracted with ether. The ether solution was washed with water, dried with calcium chloride and evaporated on a water bath. The orange-yellow residue was extracted with warm petrol ether, which removed only little, dried and dissolved in a small volume of hot alcohol. On addition of water long orange needles crystallized out. M.p. 169° C., mixed melting point with *4-amino-2.6-dinitrotoluene* of m.p. 175° C.: 172° C.

*Diaminonitro compounds.* Ether extract II was extracted with 1 p.c. hydrochloric acid. The acid solution was more deeply orange than the hydrochloride solution of 2.4-diamino-6-nitrotoluene which is a greenish yellow. The bases were brought into ether with sodium carbonate and the ether solution washed with a few ml. of water, dried over calcium chloride and evaporated. The brown-red oily residue was extracted with boiling light petroleum (b.p. 50–60° C.). On cooling the residue crystallized in long spikes. It was dissolved once more in ether, leaving a small amount of brown amorphous material behind, and dissolved in a few ml. of hot chloroform. After addition of a few drops of light petroleum and standing, orange needles crystallized out, which were recrystallized from very little chloroform. M.p. 138° C.; mixed m.p. with 2.4-diamino-6-nitrotoluene of m.p. 138° C.: 117–127° C. This large depression showed that the substances are not identical. Mixed m.p. with 5-nitrophenylene diamine (cf. below) of m.p. 145° C.: 141° C. The substance is thus *5-nitro-m-phenylene diamine.* With the exception of its smaller solubility in chloroform and the deeper colour of its solutions in HCl, it greatly resembles 2.4-diamino-6-nitrotoluene in appearance and properties; both give e.g. a strong yellow colour if nitrite is added to solutions in hydrochloric acid.

The mother liquors of the nitrophenylene diamine gave material of lower m.p., varying from 115° C to 130° C., and giving smaller or no m.p. depressions with 2.4-diamino-6-nitrotoluene. Some of this material was acetylated in pyridine with acetic anhydride. The acetyl compound was precipitated with water and recrystallized from hot water. Faintly yellow needles of m.p. 262° C. (uncorr.); mixed with 2.4-diacetyldiamino-6-nitrotoluene of m.p. 269° C.: 266° C. After acid hydrolysis the free base melted at 131° C. (corr.) and gave no m.p. depression with *2.4-diamino-6-nitrotoluene* of m.p. 138° C. $\epsilon_{sp.} = 190$, the same as that of 2.4-diamino-6-nitrotoluene (cf. below). A small part of the acetyl compound which was not dissolved in the first extraction with hot water, gave on extraction with a large volume of hot water a mixture of white needles with some yellow crystals of diacetyl nitrophenylene diamine. The low melting fractions consist, therefore, of a mixture of 2.4-diamino-6-nitrotoluene with 5-nitrophenylene diamine.

*Aminonitrocresols.* The alkali-soluble fraction of ether extract I was brought back into ether by neutralizing the HCl solution with sodium acetate. 20 p.c. hydrochloric acid extracted most of it from ether with yellow colour. The hydrochloride of the azo-dye obtained by diazotisation and coupling with naphthyl ethylene diamine was somewhat bluish red and had a maximum of absorption at 550 mμ. The alkali-soluble fractions of ether extracts II and III were fractionated with $NaHCO_3$ and hydrochloric acids of various strengths, but no crystalline material could be obtained from any of these fractions. The solutions in NaOH were deep yellow to red, while the acid solutions were almost colourless to light yellow, except the 2 p.c. HCl fraction which was a reddish yellow and evidently contained the compound of similar properties obtained from the urine of rats dosed with 2.4-diamino-6-nitrotoluene and described below.

*Metabolic Products from the Urine of Rats dosed with 2.4-diamino-6-nitrotoluene.*

A rat was fed with food containing 1·5 mg. of 2.4-diamino-6-nitrotoluene per gm. 500 ml. of urine were collected and worked up as described above. The results are given in Table 4.

TABLE 4.

*Diazotisable amines in the urine of rats dosed with 2.4–diamino–6–nitrotoluene.*

| | Σ E. | Δ Σ E. |
|---|---|---|
| Total urine | 27,000 | |
| Ether extract I | | 600 |
| NaOH extractable | 450 | |
| 1 p.c. HCl extractable | 170 | |
| 20 p.c. HCl extractable | 130 | |
| Ether extract II | | 19,600 |
| NaOH extractable | 550 | |
| 1 p.c. HCl extractable | 16,800 | |
| 20 p.c. extractable | 50 | |
| Ether extract III | | 1,300 |
| NaOH extractable | 940 | |
| 1 p.c. HCl extractable | 350 | |
| 20 p.c. HCl extractable | 10 | |
| Residual urine | 5,500 | |
| Aminonitrocresols corr. | 1,600 | |
| Aminodinitro corr. | 160 | |
| Diaminonitro corr. | 17,300 | |

As would be expected the weak-basic fraction is negligible and the major part of the diazotisable amine is found in the nitrotoluylene diamine fraction, while the aminonitrocresol fraction is also clearly increased.

Nevertheless the 2.4–diamino–6–nitrotoluene does not remain unaltered. The 1 p.c. HCl extract of the second ether extract was orange, not light yellow. When this fraction was worked up as described for the same fractions from the urine of rats dosed with T.N.T. 25 mg. crystallized from chloroform in orange needles. M.p. after recrystallization from chloroform 145° C. Mixed m.p. with 2.4–diamino–6–nitrotoluene (m.p. 138° C.): 119° C., a large depression.

Found: C, 47·03, 46·80; H, 4·59, 4·67; N, 27·3. Calculated for $C_6H_7O_2N_3$: C, 47·03; H, 4·58; N, 27·45.

The substance is therefore a nitrophenylene diamine. *5–nitro–m–phenylene diamine* has been prepared by Flürschein (1905) by reduction of symmetrical trinitrobenzene and reported to have m.p. 140–141° C.

A few mg. were dissolved in 1 ml. of pyridine and 1 ml. of acetic anhydride was added. The colour changed from orange to yellow. After 1 hour's standing water was added and the yellow precipitate filtered off. It was sparingly soluble in hot water, somewhat more in hot glacial acetic. It was dissolved in hot glacial acetic acid and two volumes of hot water added. A precipitate of yellow triangles and twin prisms was obtained. The substance became gradually black on heating over 300° C., began to decompose at 320° C. and frothed up at 336° C. Flürscheim describes the *diacetyl compound of 5–nitro–m–phenylene diamine* as light yellow crystals, not melting up to 270° C. Found: C, 50·48, 51·02; H, 4·55, 4·82; N, 17·9. Calculated for $C_6H_5O_2N_3(COCH_3)_2$: C, 50·61; H, 4·68, N, 17·7. After hydrolysis by boiling hydrochloric acid the original compound m.p. 145° C. was recovered.

The mother liquors gave further crystallization on addition of more light petroleum, which, however, had lower and unsharp melting point between 108° C. and 119° C. the last fractions melted again somewhat higher (128° C.) and gave no melting point depression with *2.4–diamino–6–nitrotoluene*. Attempts to separate the mixture by chromatography on aluminium oxide failed; a small part was adsorbed from the chloroform solution and eluted by ether; brown-orange powder, m.p. 190° C., unsharp.

The specific extinction coefficient of the low melting fractions was 210, while that of 2.4–diamino–6–nitrotoluene is 190, and of 5–nitrophenylene diamine 260 (cf. below). From this it can be concluded that the mother liquors contain about ⅓ of nitrophenylene diamine and ⅔ of nitrotoluylene diamine, and that about equal amounts of the two substances are present in urine.

The alkali-soluble fractions of the diazotisable amines consisted mainly of substances of *aminophenolic* character. $NaHCO_3$ extracted only about 6 p.c. of the total absorption and 0·5 p.c. HCl extracted from ether 13 p.c., which was probably mostly nitrotoluylene and phenylene diamines, extracted by water. The main part was extracted from ether by 2 p.c. hydrochloric acid with pinkish-orange colour. While this fraction was distinctly more uniform than any of the aminonitrocresol fractions from T.N.T.-fed rats, attempts to obtain this substance crystalline failed.

*T.N.T. Reduction Products from two Urines of Munition Workers.*

1 l. of the urine of B., containing according to the analysis 148 mg. of T.N.T. in the form of diazotisable amines, and 620 ml. of the urine of P., containing 103 mg. were worked up in the manner described above. Table 5 gives the results.

TABLE 5.

*Diazotisable amines in the urine of munition workers.*

| | B. | | P. | |
|---|---|---|---|---|
| | Σ E. | Δ Σ E. | Σ E. | Δ Σ E. |
| Total urine | 20,700 | | 14,500 | |
| Ether extract I | | 7,880 | | 4,070 |
| NaOH extracted | 1,100 | | 690 | |
| 1 p.c. HCl extracted | 20 | | 110 | |
| 20 p.c. HCl extracted | 7,470 | | 3,580 | |
| Ether extract II | | 1,750 | | 1,870 |
| NaOH extracted | 180 | | 310 | |
| 1 p.c. HCl extracted | 1,310 | | 1,160 | |
| 20 p.c. HCl extracted | 80 | | 120 | |
| Ether extract III | | 820 | | 860 |
| NaOH extracted | 200 | | 230 | |
| 1 p.c. HCl extracted | 140 | | 110 | |
| 20 p.c. HCl extracted | 30 | | 50 | |
| Aminonitrocresols corr. | 850 | | 840 | |
| Aminodinitro corr. | 7,540 | | 3,730 | |
| Diaminonitro corr. | 1,410 | | 1,340 | |

In both urines nitrotoluidines prevailed, more so in the urine of B. than of P. In the urine of B. 77 p.c. of the total absorption was due to nitrotoluidines, 14 p.c. to nitrotoluylene diamine and 9 p.c. to aminonitrocresols; in the urine of P. the corresponding figures were 63 p.c., 23 p.c. and 14 p.c.

A small part of the ether extracts I was tested for *hydroxylamino dinitrotoluene* before extraction with sodium hydroxide. In both cases (B. and P.) a purple Webster test was obtained, indicating the presence of the hydroxylamino compound. A comparison of Δ Σ E of the first ether extracts with the sum of Σ E of their fractions shows that the latter are larger, probably due to the conversion of some hydroxylamino into amino compound during the separation.

*Dinitrotoluidines.* For the isolation of the dinitrotoluidines the 20 p.c. hydrochloric acid extracts of the first ether extracts from the urines of B. and P. were worked up. The HCl extracts were overlayered with ether and neutralized with solid $Na_2CO_3$, driving the amines into the ether. Evaporation of the ether left a mixture of orange and more lightly yellow crystals of unsharp m.p. 140–163° C. They were taken up once more by ether. A few ml. of 20 p.c. hydrochloric acid extracted only part of the yellow pigment, while the remainder was extracted by 30 p.c. HCl. The fraction extracted by 20 p.c. HCl was brought back into ether by neutralization with solid $Na_2CO_3$. After evaporation, the residue of the ether solution was once extracted with boiling light petroleum (b.p. 60–80° C.) which did not dissolve much. The residue was recrystallized from chloroform with addition of light petroleum (b.p. 50–60° C.); long orange needles, m.p. 172° C. (corr.), mixed m.p. with *4–amino–2.6–dinitrotoluene* of m.p. 175° C.: 173° C.

The more weakly basic fraction was also brought into ether with $Na_2CO_3$, and the ether solution evaporated. Light petroleum (b.p. 60–80° C.) extracted from the residue lemon-yellow crystals, m.p. 130–133° C., in the form of prisms and leaflets with straight extinction under polarized light. The part not dissolved by light petroleum was crystallized from alcohol with addition of water. These crystals were more orange, but their m.p. was the same. In a second preparation, a mixture of yellow and orange crystals was obtained, m.p. 128–129° C., unsharp, completely melted at 165° C.

The analysis of the crystals, m.p. 130–136° C. gave values expected for dinitrotoluidine. Found: C, 42·86, 43·12; H, 3·61, 3·63; N, 20·6. Calc. for $C_7H_7O_4N_3$: C, 42·62; H, 3·58; N, 21·3.

Acetylation in pyridine with acetic anhydride gave a mixture of flat square-ended needles with fine colourless needles. M.p. unsharp, began to melt at 159° C., but the last remainders melted only at 197° C. *2–acetylamino–4.6–dinitrotoluene* is described by Channon *et al.* (1944) as yellow needles, melting at 159–160° C., while 4–acetylamino–2.6–dinitrotoluene forms fine almost colourless needles of m.p. 227° C.

From these observations and the analyses it can be concluded the crystals of low m.p. contained a mixture of 2–amino–4.6–dinitrotoluene with 4–amino–2.6–dinitrotoluene.

The 1 p.c. hydrochloric acid extract of the second ether extracts containing the *diaminonitro compounds* was of brown-orange colour, and therefore probably also contained nitrophenylene diamine besides nitrotoluylene diamine. It was brought into ether with sodium acetate and evaporation of the ether yielded a red-brown semi-crystalline mass. Its amount was too small to obtain the substances crystalline and to effect their separation.

At least two different *aminonitrocresols* appeared to be present in the alkali extracted fraction. Those from ether extract I yielded a weakly basic compound, extractable from ether by 20 p.c. hydrochloric acid, which on diazotisation and coupling with naphthyl ethylene diamine yielded an azo-dye hydrochloride with maximum of absorption at 525 mμ. Almost half of the alkali-soluble diazotisable amine from ether extract III could be extracted from ether by $NaHCO_3$. The other half gave an azo-dye hydrochloride with absorption maximum at 535 mμ. No crystalline material could be isolated.

TABLE 6.

*Molar extinction coefficients of the dyes obtained from T.N.T. reduction products by diazotisation and coupling with N(1–naphthyl) ethylene diamine.*

| Compound. | Conc. of amine μg. per ml. | HCl conc. p.c. | Solvent. | $\epsilon_{mol.} \times 10^{-3}$. |
|---|---|---|---|---|
| 4–amino–2.6–dinitrotoluene | 2·0 | 0·8 | water | 36·3 |
| | 4·0 | „ | „ | 38·6 |
| | 2·0 | „ | 50 p.c. alcohol | 35·2 |
| | 2·0 | „ | urine | 34·7 |
| | 4·0 | „ | „ | 32·6 |
| | 2·0 | „ | urine, alcohol | 33·1 |
| | 1·3 | 6·0 | water | 31·2 |
| | 1·3 | „ | 50 p.c. alcohol | 35·3 |
| | 6·3 | „ | „ „ | 35·3 |
| | 13·3 | „ | „ „ | 33·6 |
| | 2·0 | „ | urine | 32·7 |
| | 2·0 | „ | urine, alcohol | 35·1 |
| | 5·0 | „ | „ „ | 34·7 |
| Substance m.p. 129° C. | 4·0 | 0·8 | water | 23·1 |
| (mixture of 2–amino- and | 4·0 | 0·8 | urine | 21·8 |
| 4–aminodinitrotoluene) | 2·6 | 6·0 | water | 32·3 |
| | 5·5 | „ | „ | 23·4 |
| | 1·3 | „ | 50 p.c. alcohol | 31·1 |
| | 4·0 | „ | „ „ | 30·7 |
| | 2·6 | „ | urine | 23·2 |
| | 4·0 | „ | „ | 23·8 |
| | 6·6 | „ | „ | 23·1 |
| 2.4–diamino–6–nitrotoluene | 1·8 | 0·8 | water | 31·1 |
| | 3·8 | „ | „ | 31·1 |
| | 5·0 | „ | „ | 34·5 |
| | 7·7 | „ | „ | 31·9 |
| | 15·4 | „ | „ | 31·9 |
| | 2·0 | „ | urine | 31·7 |
| | 4·0 | „ | „ | 33·7 |
| | 5·0 | „ | „ | 32·2 |
| | 12·5 | „ | „ | 30·9 |
| | 15·4 | „ | „ | 30·2 |
| 5–nitro–*m*–phenylene diamine | 2·6 | 0·8 | water | 42·8 |
| | 5·1 | „ | „ | 41·2 |
| | 12·7 | „ | „ | 40·9 |
| | 6·3 | „ | 50 p.c. alcohol | 32·7 |
| | 1·3 | „ | urine | 41·2 |
| | 2·1 | „ | „ | 39·6 |
| | 2·6 | „ | „ | 39·3 |
| | 5·1 | „ | „ | 38·7 |
| | 12·7 | 6·0 | „ | 33·0 |
| | 2·1 | „ | water | 30·8 |
| | 2·1 | „ | urine | 31·5 |

*Specific Extinction Coefficients.*

In Table 6 data are collected on the molar extinction coefficients of the azo-dyes obtained from T.N.T. reduction products by diazotisation and coupling with N(1–naphthyl) ethylene diamine under various conditions in urine and water. The volume before diazotisation was always 5 ml., diluted by the reagents to 8 ml.; in some experiments alcohol was added afterwards, to a volume of 16 ml. The second column gives the concentration of the azo-dye in the final solution subjected to measurement, expressed in $\mu$g. T.N.T. reduction product from which the dye had been derived. The third column gives the concentration of HCl in the solution before diazotisation. Diazotisation and coupling were carried out as described in the first paper of this series. If urine was added, the urine without amine was treated in the same manner and the blank subtracted. The extinctions were measured in a Hilger Visual Absorption Spectrophotometer.

A higher HCl concentration decreases colour development from 4–amino–2.6–dinitrotoluene, but only because the dye hydrochloride is rather insoluble in strong HCl and tends to precipitate; if alcohol is added the same molar extinction coefficients are obtained. In 0·8 p.c. HCl the extinction coefficients in urine are slightly lower than in water.

The latter is also found with 2–amino–4.6–dinitrotoluene (containing some 4–amino–2.6–dinitrotoluene). With high HCl concentration the same extinctions are found in urine as with low HCl concentrations, but in water and particularly in 50 p.c. alcohol higher extinctions are found with high HCl concentration.

The extinction of the dye from 2.4–diamino–6–nitrotoluene is the same in urine and water In higher HCl concentration the dye hydrochloride is little soluble in water and even in alcohol.

5–nitro–*m*–phenylene diamine gives lower extinctions if diazotised in strong HCl than if diazotised in 0·8 p.c. HCl. The presence of urine depresses the extinction slightly. In contradistinction to the other three substances the concentration of the amine has a noticeable, though slight, effect on the extinction of the dye obtained from it, the extinction decreasing with increasing concentration of amine.

## SUMMARY.

A method was worked out by which reduction products of T.N.T. excreted in the urine can be separated into the following fractions: dinitroamino compounds, diaminonitro compounds and aminophenols.

In the urine of rats dosed with T.N.T. the diaminonitro compounds prevailed. 2.4–diamino–6–nitrotoluene and 1.3–diamino–5–nitrobenzene were isolated and the latter compound was also found in the urine of rats dosed wtih 2.4–diamino–6-nitrotoluene. The rat is thus able to remove the methyl group of the toluene nucleus. 4–amino–2.6–dinitrotoluene was isolated from the dinitrotoluidine fraction, while no evidence for the excretion of hydroxylamino compounds was found in the rat.

In two munition workers' urines dinitrotoluidines prevailed. 4–amino–2.6–dinitrotoluene and 2-amino–4–6 dinitrotoluene were found. The urines also contained hydroxylamino dinitrotoluenes (not azoxy compounds) and diaminonitro compounds. Diazotisable alkali-extractable amino compounds of phenolic character (probably aminonitrocresols) occurred in the urine of both rats and humans dosed with T.N.T.

The molar extinction coefficients of the azo-dyes produced under various conditions from reduction products of T.N.T. by diazotisation and coupling with N(1-naphthyl) ethylene diamine were measured.

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# DEPHOSPHORYLATION IN LIVER EXTRACTS

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*(Accepted for publication 27th October, 1944.)*

These investigations are concerned with the mechanism involved in the production of glucose from hexose phosphates by liver extracts. Cori and Shine (1936) and Goda (1937) have reported investigations on the transformation of fructose into glucose by rat liver slices and also the formation of glucose when the Robison ester (equilibrium mixture of hexose–6–phosphates) was dephosphorylated by liver pulp. It is concluded that glucose–6–phosphate is split by phosphatase and that, with continual re-establishment of the equilibrium between glucose–6– and fructose–6–phosphate, eventually the hexose monophosphates are hydrolysed with the production of only glucose.

Likewise the breakdown of polysaccharides by liver extracts at low phosphate concentrations yields only free hexoses. They consist of at least 97 p.c. glucose, the remainder being fructose. These observations may be correlated with the fact that in blood there is normally present little, if any, fructose.

That the breakdown of organic phosphates into their components by a non-specific phosphatase is dependent upon the $pK'_2$ of the substrate in accordance with the Michaelis–Menten equation was verified by Delory and King (1943). Liver extracts contain the enzyme isomerase (Lohmann, 1933) capable of converting at a high reaction rate, glucose–6–phosphate into the equilibrium mixture of 66 p.c. glucose–6– and 33 p.c. fructose–6–phosphate; and since the $pK'_2$ of glucose–6– and fructose–6–phosphates have been found to be identical (Meyerhof, 1930), one would expect that free glucose and fructose would be formed in the ratio 2:1.

## EXPERIMENTAL.

All the experiments were carried out with liver extracts obtained from rabbits fasted for forty-eight hours. The livers were perfused *in situ* with normal saline at 37° C., then removed and ground through muslin gauze. The pulp was extracted with twice its weight of distilled water. The suspension was shaken for five minutes and centrifuged at 3,500 revolutions per minute. The turbid, clay-coloured supernatant was diluted with an equal volume of fluid containing the substrate and other substances as set out in the Tables and Figures.

The hexose phosphates used as substrates were glucose–1–phosphate, as the potassium salt, Embden-Robison equilibrium ester and fructose–6–phosphate (Neuberg ester). The first two were prepared from starch and fresh muscle extract (Fantl and Anderson, 1941); the Embden ester was isolated as the barium salt and, prior to use, converted into the sodium salt by the addition of sodium sulphate. Fructose 1–6 diphosphate was obtained by fermentation of cane sugar by autolysed baker's yeast (Brand: "Effront") using the process of Neuberg and Lustig (1942). Fructose–6–phosphate was prepared from the diphosphate by acid hydrolysis according to Neuberg (1918) and isolated as the calcium salt which, by the addition of sodium oxalate, was converted into the sodium salt. The di-sodium phenyl and glycero-phosphates used were commercial preparations.

The estimations of glucose and glucose–6–phosphate were carried out by the technique of Somogyi and Shaffer, as modified by Harding and Downs (1933), the reducing capacity of glucose–6–phosphate being 77 p.c. of glucose. Fructose and fructose–6–phosphate were determined by the method of Roe (1934), the colour value of the latter being 48 p.c. of fructose. Inorganic phosphate was estimated colorimetrically by the method of Kuttner and Cohen (1927), and glucose–1–phosphate after ten minutes hydrolysis in 1 N. $H_2SO_4$.

[1] Working under a full time grant from the National Health and Medical Research Council.

When liver extracts, which had been kept for several days at + 6° C., were incubated with any of the substrates mentioned, no appreciable difference in the breakdown rates of the phosphate esters was detected. However, most of the extracts which had been dialysed for three to four days against normal saline or distilled water at the same temperature lost their power to split hexose-phosphates although they were still able to hydrolyse phenyl- or glycero-phosphate (Table 2).

The dialysed extracts contained phosphoglucomutase for the conversion of glucose–1–phosphate into the 6–isomer and, although the presence of inorganic phosphate rendered this enzyme inactive it could be reactivated by the addition of cysteine (Table 3).

TABLE 3.

*Influence of cysteine on phosphoglucomutase activity.*

Dialysed liver extracts incubated for 45 minutes at 37° C. with glucose–1–phosphate corresponding to 380 mg. p.c. glucose M/45 Sörensen phosphate buffer, $10^{-3}$ M. $MgSO_4$, and, in some cases, 0·03 M. cysteine or 0·07 M. glycine. Figures are calculated as mg. hexose per 100 ml. reaction mixture.

| Expt. No. | Additions. | pH. | Free hexose calculated from in inorg. P. | Formation of hexose–6–phosphates estimated by Reduction. | Disappearance of glucose–1–phosphate. |
|---|---|---|---|---|---|
| 1 | None | 6·6 | 0 | 15 | 0 |
| | Cysteine | 6·6 | 10 | 195 | 220 |
| | Glycine | 6·6 | 20 | 15 | 10 |
| 2 | None | 6·8 | 0 | 100 | 100 |
| | Cysteine | 6·8 | 0 | — | 290 |

Also added fructose–6–phosphate was readily converted into the Embden ester (Table 4) showing that the dialysed extracts contained active isomerase.

TABLE 4.

*Isomerase activity in dialysed extracts.*

Dialysed liver extracts incubated for 40 minutes at 37° C. with fructose–6–phosphate or glucose–1–phosphate corresponding to 400–500 mg. p.c. hexose, $10^{-3}$ M. $MgSO_4$ and in some cases 0·03 M. cysteine. Figures calculated as mg. hexose per 100 ml. reaction mixture.

| Expt. No. | Additions. | pH. | Free hexose calc. from inc. in inorg. P. | Hexose–6–phosphates formed estimated by Reduction. | Decrease in glucose–1–phosphate. | Fructose–6–phosphate. At start. | At end. |
|---|---|---|---|---|---|---|---|
| 1 | Glucose–1–phosphate | 8·2 | 0 | 300 | 330 | 6 | 112 |
| 2 | Glucose–1–phosphate | 7·8 | 0 | 175 | — | 2 | 65 |
| 3 | Glucose–1–phosphate | 7·2 | 10 | 280 | 280 | 4 | 112 |
| 4 | Glucose–1–phosphate + cysteine | 6·7 | 10 | 222 | 230 | 9 | 125 |
| 5 (a) | Glucose–1–phosphate | 6·3 | 10 | 90 | 94 | 6 | 38 |
| (b) | Glucose–1–phosphate + cysteine | 6·3 | 10 | 225 | 185 | 6 | 90 |
| 6 | Fructose–6–phosphate | 6·0 | 55 | | | 460 | 130 |
| | | 6·5 | 50 | | | 495 | 165 |
| | | 7·3 | 60 | | | 460 | 148 |
| | | 7·5 | 80 | | | 400 | 130 |
| 7 | Fructose–6–phosphate | 5·2 | 45 | | | 510 | 145 |
| | | 8·6 | 45 | | | 376 | 125 |

Reactivation of these dialysed extracts to obtain breakdown of hexose-monophosphates was attempted by the addition of boiled liver- or muscle extracts or ultrafiltrates of liver extracts and amino acids (Table 2, experiments 4–8). However, no satisfactory restoration of the enzyme

system could be observed and it appears that the enzyme responsible for the breakdown of glucose–6–phosphate is less stable than the non-specific phosphatases.

From these experiments we cannot decide whether glucose–6–phosphate is broken down into its components by hydrolysis or by a transphosphorylation. The possibility of transphosphorylation via adenyl-polyphosphate was tested by addition to active liver extracts of adenylic acid deaminase prepared from rabbit muscle (Schmidt, 1928), with the view in mind that the inosinic acid formed would be inferior to adenylic acid as an acceptor (Kleinzeller, 1942). However, following incubation with adenylic acid deaminase, no significant differences could be observed (Table 5).

TABLE 5.

Fresh liver extract incubated at 37° C. for 40 minutes with Embden-Robison ester* corresponding to 400 mg. p.c. hexose and in some cases the extract was incubated with adenylic acid deaminase—"A.A.D."†—before the addition of the substrate.

| Expt. No. | Additions. | Conditions for the incubation of liver extract with A.A.D. before the addition of the substrate. | pH. | Mg. p.c. inc. in inorg. P. |
|---|---|---|---|---|
| 1 | None | — | 6·6 | 33 |
| | A.A.D.$_2$ | 30 minutes at room temperature | 6·8 | 27 |
| 2 | None | — | 6·7 | 46 |
| | A.A.D.$_2$ | 45 minutes at 25° C. | 6·9 | 44 |
| 3 | None | — | 6·7 | 55 |
| | A.A.D.$_1$ | 60 minutes at 25° C. | 7·2 | 43 |
| | A.A.D.$_2$ | 60 minutes at 25° C. | 7·1 | 48 |
| 4 | None | — | 7·1 | 65 |
| | A.A.D.$_2$ | 60 minutes at 25° C. | 7·1 | 63 |

* In these experiments the equilibrium ester was purified with mercuric acetate in order to remove adenylic acid.

† Two procedures for the preparation of adenylic acid deaminase have been employed. A.A.D.$_1$ refers to the bicarbonate extract of rabbit muscle kept for three days, covered with toluene, in the refrigerator. A.A.D.$_2$ refers to a bicarbonate extract of rabbit muscle washed with isotonic saline.

These results lead, therefore, to the conclusion that glucose–6–phosphate is attacked by an enzyme which we term glucose–6–phosphatase and suggest that it is related to acid phosphatases.

Sodium fluoride inhibits glucose–6–phosphatase to the same extent as the non-specific phosphatases (Table 6).

TABLE 6.

*Inhibition of liver phosphatases by fluoride.*

Fresh liver extracts incubated for 35 minutes at 37° C. with 0·03 M. Embden-Robison ester, phenyl or glycero-phosphate and $10^{-3}$ M. $MgSO_4$ and with or without M/20 NaF.

| Expt. No. | Substrate. | pH. | P.c. inhibition by NaF. |
|---|---|---|---|
| 1 | Embden-Robison ester | 6·1 | 41 |
| | | 6·8 | 45 |
| | Phenyl phosphate | 6·6 | 39 |
| | | 7·4 | 24 |
| | | 9·8 | 9 |
| 2 | Embden-Robison ester | 5·8 | 53 |
| | | 6·1 | 50 |
| 3 | Phenyl-phosphate | 5·3 | 96 |
| | | 8·9 | 20 |
| 4 | Glycero-phosphate | 5·5 | 73 |
| | | 9·2 | 8 |

Phlorhizin inhibits the breakdown of glucose–6–phosphate to a greater extent than that of phenyl and glycero-phosphate (Fig. 3). The inhibition by phlorhizin is dependent upon the pH of the system and declines with decreased acidity.

Neither insulin, cysteine, glycine nor cyanide had any influence on the course of glucose production from hexose phosphates.

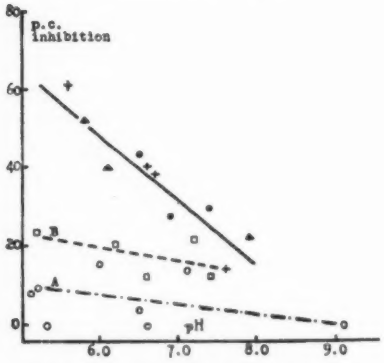


**Fig. 3.** Liver extracts incubated for 40 minutes at 37° C. with 0·01–0·03 M. substrate, $10^{-3}$ M. $MgSO_4$, and with and without M/100 phlorhizin.

Sign ▲ denotes an experiment with equilibrium ester; signs ● and +, experiments with fructose–6–phosphate. Hence the solid line represents the inhibition of hexose–6–phosphate breakdown by M/100 phlorhizin.

Sign O and the dotted line A represents the inhibition of breakdown of phenyl-phosphate and sign □ and the dotted line B the inhibition of glycerophosphate breakdown.

## DISCUSSION.

The experimental results clearly show that the breakdown of glucose–6–phosphate in liver is catalysed by a specific enzyme. Since no evidence for transphosphorylation was obtained, the observations permit only one conclusion, namely, that the formation of glucose from glucose–6–phosphate is due to hydrolysis. This assumption may be justified as follows:

The pH activity curve for the breakdown of hexose-mono-phosphates differs from that obtained for other phosphoric acid esters, such as glycero- and phenylphosphates. Although the liver extracts contained alkaline phosphatases, no second peak in the pH activity curve with hexose–6–phosphates as substrates could be demonstrated. Glucose–6–phosphatase is less stable than phosphoglucomutase, isomerase and the non-specific phosphatases as evidenced by the dialysis experiments. Glucose–6–phosphatase reacts to inhibitors in the same way as the non-specific phosphatases, sodium fluoride inhibiting to the same degree although phlorhizin inhibition of the former is greater.

The preference shown by this hexose-phosphatase for glucose–6–phosphate is remarkable in view of the fact that glucose–6– and fructose–6–phosphates have identical $pK'_2$. This is further evidence for the specificity of this enzyme.

The question of the direct breakdown of glucose–1–phosphate into its components has been discussed again by Mann and Lutwak-Mann (1944). There is no experimental evidence to support this assumption. It is established that in liver extracts no breakdown of glucose–1–phosphate occurs below pH6 although the hexose–6–phosphates are split, whereas above pH6 the mechanism is obscured by the activity of phosphoglucomutase.

In preliminary experiments using zinc ions as inhibitors it was shown that breakdown of the glucose–1–phosphate is inhibited to a greater extent than that of glucose–6–phosphate. Further, on theoretical grounds, it is unlikely that glucose–1–phosphate is split by phosphatases since it is a glucoside and not an ester.

Glucose–6–phosphatase does not appear to be identical with enzymes described by other workers. For instance, Gomori (1943) has shown that hexose-diphosphatase, an enzyme isolated from autolysed tissue, is specific for the hydrolysis of fructose–1–6–diphosphate and has an optimum activity at pH9, being inactive in the physiological range. Thus glucose–6–phosphatase differs from Gomori's enzyme in its lability and activity in the intracellular pH zone.

SUMMARY.

Fresh extracts of rabbits' livers contain more acid phosphatase than alkaline phosphatase as judged by the hydrolysis of phenyl- and glycero-phosphate. In addition to the nonspecific phospho-monoesterases the extracts contain an enzyme catalysing the breakdown of glucose–6–phosphate into its components. This enzyme has been called glucose–6–phosphatase, and has its optimum activity between pH6 and 7. Glucose–6–phosphatase disappears after prolonged dialysis at low temperatures, whereas the non-specific phosphatases, phosphoglucomutase and isomerase are stable under the same conditions. The inhibition of glucose–6–phosphatase by phlorhizin is greater than that of the non-specific phosphatases. Phosphoglucomutase in dialysed extracts which have been inactivated by inorganic phosphate can be reactivated by the addition of cysteine.

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# THE INFLUENCE OF TEMPERATURE ON THE DEVELOPMENT OF THE DIFFERENT STAGES OF *CALANDRA ORYZAE* L. AND *RHIZOPERTHA DOMINICA* FAB. (COLEOPTERA).

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*(Accepted for publication 24th November, 1944.)*

In the experiments described in this paper the rate of development of the different stages of *C. oryzae* and *R. dominica* has been determined at different temperatures. The experiments also established the range of temperature within which each stage of these insects can complete its development.

## EXPERIMENTAL TECHNIQUE.

The development of the different stages of *C. oryzae* and *R. dominica* was followed within single whole grains of wheat, since these insects normally complete their development from the egg to the adult stage inside cereal grains. The eggs required for the experiments were obtained from laboratory cultures as described in a previous paper (Birch, 1944a). Single individuals of *C. oryzae* were established in each grain by ensuring that only one viable egg was present in each grain (Birch, 1944b). More difficulty was experienced with *R. dominica* owing to the fact that the eggs are laid outside the grain and the first stage larva wanders about freely before it enters the grain. When eggs of *R. dominica* placed singly on grains of wheat spaced in a dish were incubated at favourable temperatures, 50 p.c. of the larvae which hatched entered the grains on which they were placed, the rest wandered away and died. When, however, the egg was confined with a single grain in a cell slightly larger than the grain, 70 p.c. of the larvae which hatched entered the grain. This procedure was followed for obtaining a number of grains each containing one developing insect of known age. The special cells used were moulded the shape of wheat grains in porous pottery discs $\frac{1}{4}$" deep.[2] Thirty cells, each slightly larger than a grain of wheat, were moulded on one disc 3" in diameter. Discs of this size could be packed neatly in No. 45 Fowler jars. The surface was sufficiently smooth to enable cover slips to fit over the cells so that larvae could not escape. The slabs were conditioned over sulphuric acid at the desired relative humidity of the experiments. Wheat grains of known moisture content were enclosed in the cells, together with one egg, by placing cover slips over the top. The discs were then packed in Fowler jars at the appropriate relative humidity. Cover slips were removed after the hatched larvae had either entered the grain or had died. Newly laid eggs of *R. dominica* could not be handled without damage, for this reason all the eggs used were incubated at the temperature of the experiment, until the cuticle had formed (3 days at most temperatures); they were then readily separated by needles and transferred on a camel's hair brush to the crease of the grain.

The development of *R. dominica* inside the grain was followed by dissecting small "windows" in the grains. The end points of the larval, prepupal and pupal stages were determined by daily examinations through the "window" with a binocular microscope. The exsertion of the head capsule of the larva was taken as the end point of the larval stage (Murray and Tiegs, 1935). Two hundred hatched eggs were used for each treatment.

With *C. oryzae* the procedure of observing the development of the different stages through a hole in the grain was not practicable, and so a "serial" method was used. A large supply of grains, each of which contained one egg not more than 14 hours old was kept at each temperature

[1] The author worked with the aid of a research grant from the Federal grant to the University of Adelaide.

[2] Plaster of Paris slabs were tried first but proved unsuitable as the first stage larva sometimes burrowed into the plaster instead of into the grain of wheat. The porous pottery slabs were made in the following way. A white Adelaide pottery clay was mixed to a fluid consistency and poured into Plaster of Paris moulds. As the clay dried out more was added until a slab of sufficient thickness was obtained. While the clay was plastic cells were moulded in it with wheat grains which had been swollen by soaking in water. The slab was dried at room temperature, the grains removed and the surface ground smooth on sandpaper. A hard but porous pottery was produced by baking at 1,150° C. for 3 hours. I am indebted to Dr. T. J. Marshall for his advice in the making of these slabs.

of the experiment. At day intervals one hundred grains were withdrawn at random; these were fixed in Carl's fixative and stored in 70 p.c. alcohol until they were dissected at a later date to determine the stage of the insects inside. About 2,500 grains, each containing one egg, were required for each temperature treatment.

Experiments in which the length of the larval, prepupal and pupal stages were determined were carried out at 70 p.c. R.H. The wheat was previously conditioned to its equilibrium moisture at 70 p.c. R.H., namely 14 p.c.[3]

## THE MORTALITY RATE AT DIFFERENT TEMPERATURES IN WHEAT OF 14 p.c. MOISTURE CONTENT.

The limiting temperatures below and above which complete development from egg to adult no longer took place were 15·2° C. and 34° C. for *C. oryzae* and 18·2° C. and 39° C. for *R. dominica* (Table 1). At 15·2° C. the development of *C. oryzae* was so slow that after 250 days when four adults had emerged the grains were transferred to an incubator at 30° C. All the insects which were alive on the 250th day at 18·2° C. gave rise to adults at 30° C. within a few days. At 18·2° C. only 2 p.c. of *R. dominica* developed to final instar larvae; these died after 230 days without becoming pupae.

TABLE 1.

*Showing the mortality of* C. oryzae *and* R. dominica *during development from egg to adult in wheat of 14 p.c. moisture content.*

| *Calandra oryzae.* | | | *Rhizopertha dominica.* | | |
|---|---|---|---|---|---|
| | P.c. mortality. | | | P.c. mortality. | |
| Temp. °C. | Egg. | All stages. | Temp. °C. | Egg. | All stages. |
| 13·0 | 99 | 100 | 18·2 | 8 | 100 |
| 15·2 | 31 | 75* | 22·0 | 0 | 53 |
| 18·2 | 10 | 12 | 26·0 | 0 | 30 |
| 25·4 | 7 | 7 | 30·0 | 0 | 26 (11)† |
| 29·1 | 8 | 10 | 34·0 | 0 | 22 |
| 32·3 | 13 | 15 | 36·0 | 0 | 31 |
| 33·5 | — | 75 | 38·2 | 0 | 86 (23) |
| 34·0 | 20 | 97 | 38·6 | 19 | 100 (83) |
| 34·6 | 100 | 100 | 39·0 | 100 | 100 (100) |

Significant differences: *C. oryzae.* At P = 0·01, egg stage = 6, all stages = 10.
*R. dominica.* At P = 0·01, egg stage = 17, all stages = 12.

* At 15·2° C. 4 p.c. emerged in a mean time of 216 days. The remaining 21 p.c. emerged when incubated at 30° C.

† Figures in parenthesis are mortality rates when the grain was damaged with a scalpel cut; this facilitated the entry of the first stage larva.

Apart from the mortality of eggs, mortality of the different stages was practically confined to the first stage larvae in both insects; for this reason no distinction has been made in Table 1. The higher mortality of first stage larvae of *R. dominica* is probably due to the extra hazard the insect has to face because its eggs are laid outside the grain. Between 26° C. and 36° C. all the mortality of the first stage larvae occurred after the larvae had entered the grain; at higher and lower temperatures about one-third of the mortality occurred before the larvae succeeded in entering the grain. When the grain was damaged with a deep scalpel cut the mortality of first stage larvae was less (Table 1), i.e. more succeeded in establishing themselves in the grain.

## THE RATE OF DEVELOPMENT AT DIFFERENT TEMPERATURES IN WHEAT OF 14 p.c. MOISTURE CONTENT.

*Calandra oryzae.*

There was no significant difference in the length of development of the same stages of the two sexes, therefore no distinction is made in Table 2.

The larva develops in the shortest time at 29·1° C.; this was three degrees lower than the temperature at which the egg stage developed in the shortest time (Table 2). The length of the prepupal and pupal stages was determined with less accuracy because these stages are much shorter in duration; observations could only be made at the same time interval for all stages,

[3] In accordance with the practice in the wheat trade, moisture contents referred to in this paper are expressed as a percentage of the wet weight.

namely 24 hours. The differences in length of development of these stages at 25·4, 29·1 and 32·3° C. shown in Table 2 are not significant. The adult remains some time in the grain while the cuticle hardens and darkens before it emerges (Column 7, Table 2).

TABLE 2.

*Showing the days required for the development of the different stages of* C. oryzae *in wheat of 14 p.c. moisture content at a density of one insect per grain.*

| Temp. °C. | Egg. | Larva. | Prepupa. | Pupa. | Egg to adult. | Days adult remains in grain. |
|---|---|---|---|---|---|---|
| 15·2 | 18·4 | | | | 216† | 14·0 |
| 18·2 | 10·4 | 62·4 | 3·7 | 17·3 | 93·8 | 14·0 |
| 25·4 | 4·3 | 18·4 | 2·0* | 6·3* | 31·1 | 3·0 |
| 29·1 | 3·6 | 13·1 | 2·0* | 6·0* | 24·7 | 3·0 |
| 32·3 | 3·3 | 16·1 | 1·8* | 4·6* | 25·8 | 2·5 |
| 33·5 | 25 p.c. emerged from the grain in a mean time of 64 days. | | | | | |

† Figure based on four insects only.

Significant differences. The data in the Table were obtained by a "serial" method (see text); its analysis was for this reason complex and no single figure can be given for a significant difference. Individual pairs have been compared, however, and all pairs adjacent vertically are significantly different except those marked with an asterisk (*).

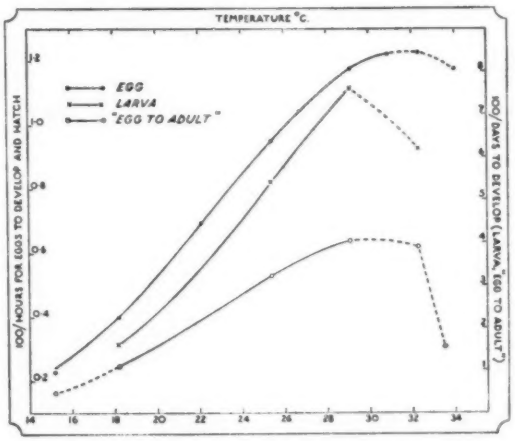


**Fig. 1.** Showing the reciprocal of the time required for the development of the different stages of *Calandra oryzae* plotted against temperature. Solid lines are the calculated curves, having the form:

Egg $$y = \frac{1 \cdot 4474}{1 + e^{5 \cdot 05828 - 0 \cdot 22468x}}$$

Larva $$y = \frac{10 \cdot 1}{1 + e^{6 \cdot 31127 - 0 \cdot 25467x}}$$

"Egg to adult" $$y = \frac{4 \cdot 8}{1 + e^{5 \cdot 99285 - 0 \cdot 26358x}}$$

The mean value of the reciprocals of the time required for the development of the larva and "egg to adult" are shown in Fig. 1. The curve for the egg stage is also shown (from Birch, 1944c). Logistic curves have been fitted to the ascending part of the curves (between 18·2° C. and 29·1° C.). Care is necessary in interpreting apparent differences between the reciprocal curves of the "larva" and "egg to adult" which are plotted on the same scale. The difference in steepness and the acuteness of the angle at the apex are expressions of a mathematical property of the reciprocals of a set of numbers of low magnitude, compared with a set of numbers of higher magnitude.[4] The difference in slope between the ascending and descending parts of the curve is, however, a characteristic of the stage concerned. The difference between the curve for the larva and that for "egg to adult" in this respect is due to the fact that larva on the one hand and the egg, prepupa and pupa on the other have different "peak" temperatures. Temperatures higher than 29·1° C. do not have the same retarding effect on the speed of development for the stages of shorter duration (Table 2).

[4] If any chosen temperature difference gives the same *percentage* difference in the time for a short stage to develop as for a stage of longer duration, then the influence of temperature is the same on a percentage basis. But the reciprocal curve for the short stage will necessarily be steeper than that for the stage of longer duration; and if the relation holds on both sides of the "temperature velocity" curve the angle at the apex will be more acute for the stage of shorter duration.

The influence of the number of insects per grain on the rate of development of *C. oryzae* was determined by comparing the rate of development with three eggs per grain with the rate at a density of one egg per grain (Table 3).

TABLE 3.

*Showing the days required for the development of the different stages of* C. oryzae *in wheat of 14 p.c. m.c. at a density of three insects per grain.*

| Temp. °C. | Egg. | Larva. | Prepupa. | Pupa. | Egg to adult. |
|---|---|---|---|---|---|
| 18·2 | 10·4 | 90·0 | 6·0 | 12·0 | 118·4 |
| 25·4 | 4·3 | 31·3 | 3·9 | 4·7 | 44·2 |
| 29·1 | 3·6 | 27·5 | 1·7 | 2·7 | 35·5 |

At the three temperatures of the experiment the time required for the development of all stages except the egg stage was longer at the higher density. In most cases only one insect survived to the adult stage in each grain. When two larvae met inside the grain one generally devoured the other; in most cases they did not meet until quite large so that by then the greater part of the grain had been destroyed. In only one case three adults emerged from one grain. In dense infestations as many as 15 eggs are laid in one grain but rarely more than one survives to the adult stage.

*Rhizopertha dominica.*

There was no significant difference in the rates of development of males and females; figures for the two sexes were combined in presenting Table 4.

TABLE 4.

*Showing the days required for the development of the different stages of* R. dominica *in wheat of 14 p.c. moisture content.*

| Temp. °C. | Egg. | Larva. | Prepupa. | Pupa. | Egg to adult. |
|---|---|---|---|---|---|
| 22·0 | 15·2 | 56·4 | 2·3 | 10·0 | 83·9 |
| 26·0 | 9·0 | 27·6 | 1·4 | 5·9 | 43·9 |
| 30·0 | 6·0 | 21·0 | 1·0* | 4·5 | 32·5 |
| 34·0 | 4·8 | 15·8 | 1·0* | 3·1* | 24·7 |
| 36·0 | 4·5 | 20·1 | 0·7* | 3·5* | 28·8 |
| 38·2 | 4·9 | 27·5 | 0·8* | 3·3* | 36·5 |

At $P = 0{\cdot}01$ all differences between means vertically adjacent are significant except those marked with an asterisk (*).

The mean reciprocals of the days for the development of the larva and "egg to adult" were plotted against temperature (Fig. 2). The curve for the egg stage is also shown (from Birch and Snowball, 1945); it has been shown previously that a logistic curve describes the trend of these points between 18·2° C. and 36·0° C. For the larva and "egg to adult", however, the logistic curve could only be fitted to points between 26·0° C. and 34·0° C. At 34° C. complete development of the larva and "egg to adult" took place in the shortest time, i.e. the peak of the curve occurs at 34° C. (2° lower than the peak temperature of the egg stage). The next temperature below 26° C. at which the experiments were carried out was 22° C.; mortality at 22° C. was almost twice that at 26° C. (Table 1); this harmful effect of low temperature may account for the departure of this point from the logistic trend of the other points. The logistic formula describes the trend of the ascending part of the "temperature velocity curve" for the egg, the larva and the "egg to adult" of both insects, within the range of temperatures at which mortality is uniformly low (Figs. 1 and 2 and Table 1).

*Comparison of the "temperature-velocity" curves for the complete development of* C. oryzae *and* R. dominica.

The limiting temperatures for the development of *C. oryzae* and *R. dominica* were 15·2–34° C. and 18·2–39° C. respectively, i.e. ranges of 19° and 21°, but on a different part of the temperature scale; *R. dominica* may be referred to as a "high-temperature insect" relative to *C. oryzae*. The range of temperatures within which mortality was low and beyond which it increased significantly were 18·2°–32·3° C. for *C. oryzae* and 26·0°–38·2° C. for *R. dominica*, a range of 14° and 12° respectively. (The difference between the magnitude of these ranges may

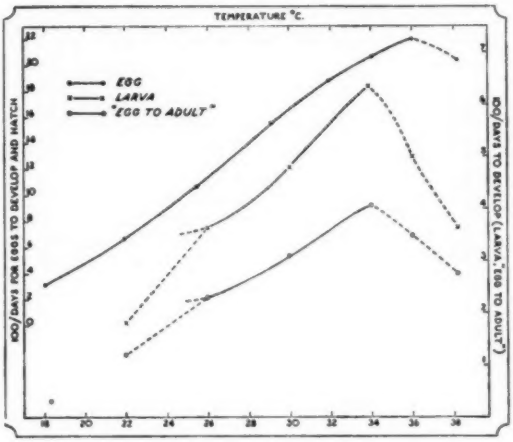


**Fig. 2.** Showing the reciprocal of the time required for the development of the different stages of *Rhizopertha dominica* plotted against temperature. Solid lines are the calculated curves, having the form:

$$\text{Egg} \qquad y = \frac{24{\cdot}98689}{1 + e^{5{\cdot}84774 - 0{\cdot}21784x}}$$

$$\text{Larva} \qquad y = 3{\cdot}25 + \frac{3{\cdot}8}{1 + e^{14{\cdot}19830 - 0{\cdot}46006x}}$$

$$\text{"Egg to adult"} \qquad y = 2{\cdot}0 + \frac{2{\cdot}5}{1 + e^{13{\cdot}72710 - 0{\cdot}44837x}}$$

The value for "egg to adult" at 18·2° C. was obtained from an experiment at alternating temperatures.

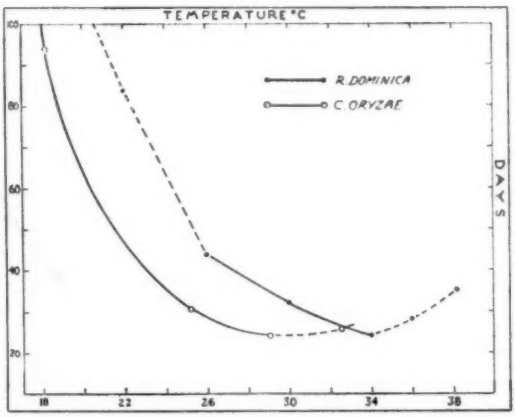


**Fig. 3.** Showing the relation between temperature and the time for the completion of development of *C. oryzae* and *R. dominica* from egg to adult. The solid lines are calculated curves being the reciprocal of those shown in Fig. 1 and Fig. 2.

be due to the limitations of the experiment, as the experiments were carried out at four degree intervals over the low part of the temperature scale). With *R. dominica* 33·5 p.c. of this range occurs above the peak temperature compared with a figure of 16·5 p.c. for *C. oryzae*. It is interesting to compare this with the average of 10–17 p.c. for the egg stage of many insects (Davidson, 1942). This fundamental difference between *C. oryzae* and *R. dominica* is clear from an inspection of Figs. 1 and 2. The greater range of *R. dominica* above the peak temperature may be associated with a shorter range below the peak temperature which follows a logistic trend. For this range to be of the same magnitude as that for *C. oryzae* (85 p.c. of the total) it would be necessary for a point at 23·5° C. to fit the logistic trend. No figure is available at this temperature. A further difference between the curves of the two insects is that it was possible to obtain an estimate of the time for the development of *R. dominica* at 22° C., a temperature below the range at which observed values fit the logistic curve.

At any temperature up to 32° C. all the stages of *R. dominica* took longer to develop than those of *C. oryzae* at the same temperature (Fig. 3). At 32·3° C. the two insects completed development from egg to adult in about the same time; this is shown by the intersection of the curves in Fig. 3 at 32·3° C. Moore (1942) found a similar relation between the rate of development of "cold climate" frogs and "warm climate" frogs; those adapted to live at lower temperatures developed faster at any particular temperature than those adapted to higher temperatures.

### *The Effect of Time of Exposure and Stage of Development on the Development of* R. dominica *at extreme High and Low Temperatures.*

At 18·2° C. *R. dominica* did not complete its development although some developed to the final instar stage. An estimate of the rate of development at 18·2° C. was obtained from the

following experiment. Larvae were established in grain by incubating newly laid eggs with grains for 9 days at 30° C.; these were transferred to 18·2° C. and for every 15 days spent at 18·2° C. one day was spent at 30° C. After 118 days had been spent at 18·2° C. the insects were kept at 30° C. until all had either emerged as adults or died. Thirty-two p.c. emerged, the remainder dying at various stages. The known mean rate of development per day of the insect at a constant temperature of 30° C. was applied to the time spent at that temperature (21 days); an estimate of 0·3 p.c. development per day at 18·2° C. was thereby obtained. This gives a hypothetical time for completion of development at 18·2° C. of 333 days.

Practically all the mortality at constant high and low temperatures occurred in the first larval instar. The influence of these temperatures on the later instars was determined in the following experiment. Larvae were established in grain of 14 p.c. moisture content at 34° C. and then incubated at this temperature for varying proportions of the full developmental period; the rest of their incubation was done at one of the extreme temperatures shown in Table 5.

TABLE 5.

*Showing the influence of extreme high and low temperatures on the development of stages of* R. dominica *other than the first instar.*

| P.c. of total developmental period spent at 34° C. | Temp. °C. at which remaining of development was spent. | P.c. mortality. |
|---|---|---|
| 90 (22 days) | 18·2 | 0 |
| 45 (11 days) | 18·2 | 90 |
| 45 (11 days) | 38·2 | 8 |
| 32 (8 days) | 38·2 | 37 |
| 65 (16 days) | 40·0 | 99* |
| 32 (8 days) | 40·0 | 100 |

* 11 p.c. developed to pupae, 15 p.c. developed to adults but died without emerging from the grain.

Table 5 shows that instars later than the first developed to some extent at temperatures at which the first instar was unable to establish iself in the grain. At 18·2° C., 38·2° C. and 40° C. temperature had a harmful effect which was proportional to its duration.

*The Rate of Development of* C. oryzae *and* R. dominica *in Wheat drier than 14 p.c. m.c.*

The influence of wheat drier that 14 p.c. on the rate of development from egg to adult is shown in Table 6. Determinations at moisture contents lower than 11 p.c. were not practicable with *C. oryzae* because of the high mortality. The combinations of temperature and moisture content of wheat at which figures are given for *R. dominica* were the driest conditions at which there was sufficient survival to give a reliable figure.

TABLE 6.

*Showing the increased number of days required for the development from the egg to the insects' emergence from the grain at low moisture levels compared with the time in wheat at 14 p.c. m.c.*

| *Calandra oryzae.* | | *Rhizopertha dominica.* | |
|---|---|---|---|
| Temp. ° C. | 11 p.c. m.c. | | |
| 18·2 | + 4·7 | 26° 11 p.c. m.c. + 9·9 | 30° 10 p.c. m.c. + 14·4 |
| 25·4 | + 2·4 | 36° 10 p.c. m.c. + 9·6 | 36° 11 p.c. m.c. + 4·2 |
| 29·1 | + 2·4 | 38·2° 12 p.c. m.c. + 12·0 | |

SUMMARY.

The ranges of temperature below and above which complete development from egg to adult was unable to take place in grain of 14 p.c. moisture content were 15·2°–34° C. for *Calandra oryzae* and 18·2°–39° C. for *Rhizopertha dominica*. Most of the mortality at unfavourable temperatures occurred in the first larval instar.

*C. oryzae* developed from egg to adult in the shortest time at 29·1° C.; the corresponding temperature for *R. dominica* was 34° C. The corresponding temperatures for the egg stages were higher than those for the larva. There was no significant difference in the rate of development of the two sexes.

Of the temperature range of *R. dominica*, 35·5 p.c. occurs above the temperature at which development is fastest (34° C.). For *C. oryzae* the figure is 16·5 p.c.

At any particular temperature all stages of *R. dominica* took longer to develop than those of *C. oryzae* except at 32·3° C., which is the highest temperature at which *C. oryzae* develops without high mortality; at 32·3° C. the two species took about the same time to develop from egg to adult.

The rate of development of *C. oryzae* was slower at a density of three insects per grain compared with a density of one insect per grain. Development was also slower in wheat drier than 14 p.c. moisture content.

*Acknowledgments.* Acknowledgment is made to Professor Davidson and Mr. H. G. Andrewartha for their helpful advice and criticism.

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# THE DEVELOPMENT OF THE EGGS OF *RHIZOPERTHA DOMINICA* FAB. (COLEOPTERA) AT CONSTANT TEMPERATURES

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The time required for the development of the eggs of *Rhizopertha dominica* Fab. has been determined at eight constant temperatures.

The experiment was designed so that the influence of moisture was kept constant over the temperature range studied. It was postulated that the influence of moisture on the rate of development would be constant at each temperature when the eggs lost the same amount of water. Since the evaporating power of the air is proportional to the product of saturation deficit x time, it was further postulated that the same amount of water would be lost at different temperatures if the product were maintained constant. The thesis was tested by using two levels of moisture at each temperature and determining the interaction between temperature and moisture according to the method developed by Birch (1944).

## MATERIALS AND METHOD.

Stock cultures of the insects were kept in wheat of 14 p.c. moisture content at 33° C. Eggs were obtained by burying thin cork discs mounted on pins, in cultures of adults one to four weeks old. After six hours the discs were removed and examined for eggs; damaged eggs were discarded. The remaining eggs were incubated *in situ* on the cork discs; 200 were used for each treatment. They were incubated in Fowler jars. The relative humidity was controlled by sulphuric acid solutions. The rapid readjustment of the relative humidity after the jar had been opened was ensured by the operation of an internal fan.

TABLE 1.

*Showing the plan of the experiment based on an approximate estimate of the duration of development of the eggs.*

| Temp. °C. | Approximate days to develop. | RH. at $M_1$ ($M_1 = 2 \cdot 68$). | RH. at $M_2$ ($M_2 = 1 \cdot 40$). |
|---|---|---|---|
| 18·1 | 34·2 | 88 | 94 |
| 22·0 | 15·0 | 77 | 88 |
| 25·5 | 10·5 | 74 | 87 |
| 29·1 | 6·6 | 66 | 82 |
| 32·0 | 5·4 | 65 | 82 |
| 34·1 | 4·5 | 63 | 81 |
| 36·0 | 4·5 | 67 | 83 |
| 38·3 | 5·0 | 73 | 86 |

Eight temperatures in the range 18·1° C.–38·3° C. were used. Temperature was controlled to ± 0·1° C. at all temperatures except 38·3° C. At this temperature control was ± 0·25° C. The two levels of moisture ($M_1$ and $M_2$) at each temperature correspond to values of saturation deficit (s.d.) × time of 2·68 and 1·40; where saturation deficit = inches of mercury and time = duration of development of the eggs in days. In a preliminary experiment it was found that the zone of highest survival of eggs lay between values of s.d. × time of 1·0 and 3·5. The two levels of moisture were chosen to lie within these limits. The relative humidities corresponding to the two moisture levels at the eight temperatures were calculated from an approximate estimate of the

[1] The authors worked with the aid of a research grant from the Federal grant to the University of Adelaide and a Hackett research grant from the University of Western Australia respectively. The second author carried out the experimental work, and in his absence owing to enlistment in the Forces, the first author did most of the statistical work and the editing of the paper.

"time" factor (Table 1). This was determined at 73 p.c. R.H. at each temperature except 18·1° C., 22·0° C. and 34·1° C. At these three temperatures a relative humidity of 90 p.c. was used.

The actual values of saturation deficit × time in the experiment were only approximately constant over all the temperatures because the first estimate of "time" was approximate. The mean deviations of these values were, however, small. At $M_1$ the per cent. mean deviation and range were 4 and 20 respectively. At $M_2$ the corresponding figures were 4 and 21.

The fan inside the jars was operated three times per day at each temperature except 18·1° C. At this temperature it was operated twice per day. The total time of operation of the fan was 6 p.c. of the incubation period of the eggs at each temperature.

The number of eggs hatched was counted at eight-hour intervals at all temperatures except 18·1° C. At this temperature the interval was twelve hours. The loss of information in estimating the mean with the group intervals used is shown in column 4 of Table 2.

## RESULTS.

### TABLE 2.

*Showing the mean duration of development at eight temperatures and two levels of moisture.*

| Temperature. ° C. | Duration of development in days. $M_1$. | $M_2$. | P.c. loss of information in estimation of mean days. | P.c. mortality. $M_1$. | $M_2$. |
|---|---|---|---|---|---|
| 18·1 | 32·09 (95)* | 33·05 (94)* | 1·4 | 19 | 54 |
| 22·0 | 15·26 (175) | 15·23 (172) | 0·2 | 16 | 13 |
| 25·5 | 9·74 (181) | 9·40 (179) | 4·8 | 14 | 11 |
| 29·1 | 6·59 (194) | 6·45 (192) | 10·6 | 7 | 5 |
| 32·0 | 5·38 (222) | 5·32 (220) | 18·8 | 3 | 10 |
| 34·1 | 5·07 (177) | 4·83 (175) | 15·9 | 9 | 23 |
| 36·0 | 4·70 (201) | 4·54 (198) | 32·5 | 8 | 23 |
| 38·3 | 5·03 (183) | 4·92 (180) | 7·0 | 20 | 12 |

* Figures in parenthesis are the number of individuals contributing to each mean.

At P = 0·01 significant differences in the time to complete development are given by:

$$d = 2{\cdot}576 \sqrt{\frac{0{\cdot}0004472 \times (N_1 + N_2)}{N_1 \times N_2}}$$

where d = significant differences on the logarithmic scale.

$N_1$ and $N_2$ = number of individuals contributing to each mean value being compared. The frequencies shown in parenthesis above have been adjusted on a proportional basis to make the analysis orthogonal. The adjusted values do not differ significantly from the originals except at 18·1° C. at $M_1$. Here it was necessary to discard at random 75 observations out of 170.

The mean values for time in days shown in the table are geometric means. The greatest deviation of any of them from the corresponding arithmetic mean is 0·6 p.c. and can be ignored.

The time for the development of the eggs was significantly shorter at the higher moisture level at all temperatures except 18·1° C., 22° C. and 32° C. At 36° C. the eggs developed in the shortest time.

*Interaction.*

There was an overall interaction between temperature and moisture. An analysis of the components of interaction shows that it was significant between nine out of the possible twenty-one combinations of temperature. The magnitude of the interaction can be found by comparing the ratios in Table 3. For complete independence these ratios would have the same value. This result shows that the use of the product saturation deficit × time as an index for maintaining constant moisture conditions is not perfect as it is not completely independent of the influence of temperature. It is probably, however, the best index that can be used.

### TABLE 3.

*Showing the components of interaction between temperature and moisture.*

| Temperature °C. | Ratio $\frac{\text{Mean time } M_1}{\text{Mean time } M_2}$. | $N_1$. | $N_2$. |
|---|---|---|---|
| 18·1 | 1·0300 | 90 | 94 |
| 22·0 | 1·0015 | 170 | 172 |
| 25·5 | 1·0362 | 181 | 179 |
| 29·1 | 1·0215 | 194 | 192 |
| 32·0 | 1·0115 | 222 | 220 |
| 34·1 | 1·0483 | 177 | 175 |
| 36·0 | 1·0341 | 201 | 198 |

Significant differences at P = 0·01 are given by:

$$d = 2{\cdot}75\sqrt{\frac{s}{N_1}+\frac{s}{N_2}+\frac{s}{N_1^1}+\frac{s}{N_2^1}}$$

where d = significant differences on the logarithmic scale.

s = 0·0004472.

$N_1$ $N_2$ $N_1^1$ $N_2^1$ = number of individuals contributing to the value of the four means involved.

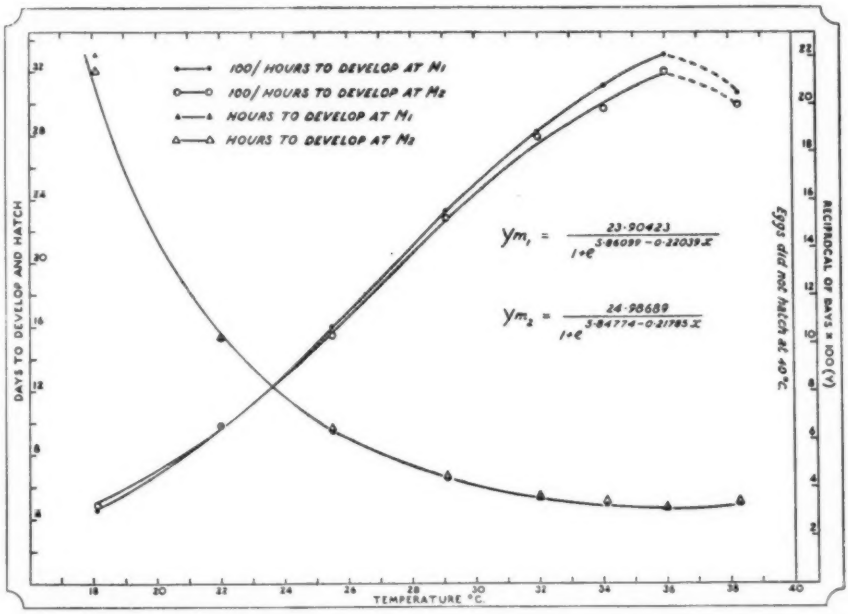
100/ HOURS TO DEVELOP AT M1
100/ HOURS TO DEVELOP AT M2
HOURS TO DEVELOP AT M1
HOURS TO DEVELOP AT M2
DAYS TO DEVELOP AND HATCH
RECIPROCAL OF DAYS x 100 (y)
Eggs did not hatch at 40°C.
TEMPERATURE °C.

**Fig. 1.** Calculated curves showing the trend with temperature of the reciprocal values of time to develop (× 100). The parts of the curves above the peak temperature were drawn free-hand. $M_1$ = moisture level corresponding to a value of saturation deficit × days = 2·68. $M_2$ = 1·40. The mean observed values for days to develop and hatch are also shown; the corresponding calculated curve is shown for $M_1$ only, as the curves for the two levels of moisture almost superimpose.

*The "Temperature-Velocity" Curve.*

In Fig. 1 the mean values of the days to complete development and the mean values of the reciprocals of days to complete development have been plotted against temperature. Logistic curves have been fitted to the reciprocal data over the temperature range 18·1° C. to 36·0° C. by the method described in a previous paper (Birch, 1944). The calculated curves for the two levels of moisture have the form:

$$\text{Moisture level 1} \quad y = \frac{23{\cdot}90423}{1 + e^{5{\cdot}86100 - 0{\cdot}22039x}}$$

$$\text{Moisture level 2} \quad y = \frac{24{\cdot}98689}{1 + e^{5{\cdot}84774 - 0{\cdot}21784x}}$$

The goodness of fit of the calculated curves is shown by comparing the difference between the observed and calculated values with the standard deviation of the observed values (Table 4). One figure for error variance cannot be provided since the variance within temperature arrays is not the same at each temperature. The $\chi^2$ test outlined in a previous paper (Birch, 1944) provides

a comprehensive test of the goodness of fit. The $\chi^2$ values for the two curves are 1·998 and 3·609. These lie between $P = 0{\cdot}50$ and $P = 0{\cdot}10$ showing that the logistic curves of the formulae given faithfully describe the trend of the curves. Except for the lowest temperature, the maximum difference between observed and calculated values on the day's scale is $\frac{1}{10}$th day.

TABLE 4.

*Showing the goodness of fit of the calculated and observed values of the reciprocal of days to develop (× 100).*

| Temp. °C. | Moisture level 1. Observed. | Calculated. | Observed-calculated. | Standard deviation. | Moisture level 2. Observed. | Calculated. | Observed-calculated. | Standard deviation. |
|---|---|---|---|---|---|---|---|---|
| 18·1 | 3·119 | 3·187 | − 0·068 | 0·1189 | 3·028 | 3·238 | − 0·210 | 0·1133 |
| 22·0 | 6·560 | 6·371 | + 0·189 | 0·2601 | 6·571 | 6·452 | + 0·119 | 0·2864 |
| 25·5 | 10·277 | 10·518 | − 0·241 | 0·3476 | 10·656 | 10·678 | + 0·022 | 0·5218 |
| 29·1 | 15·193 | 15·172 | + 0·021 | 0·7526 | 15·516 | 15·504 | + 0·012 | 0·7261 |
| 32·0 | 18·589 | 18·335 | + 0·254 | 0·7362 | 18·807 | 18·855 | − 0·048 | 0·8625 |
| 34·1 | 19·768 | 20·067 | − 0·298 | 1·0487 | 20·726 | 20·722 | + 0·004 | 1·1753 |
| 36·0 | 21·314 | 21·234 | + 0·080 | 1·1720 | 22·025 | 21·995 | + 0·030 | 0·9621 |
| | | | − 0·063 | | | | − 0·069 | |

SUMMARY.

The time for the development of eggs of *Rhizopertha dominica* has been obtained for eight temperatures at two levels of moisture.

The product saturation deficit x time for development was kept constant for all temperatures. This is the best index for maintaining constant moisture conditions over a range of temperatures. The index is not completely independent of temperature. This was shown by the interaction between temperature and the product saturation deficit x time. This interaction was, however, small.

The temperature at which eggs developed at the fastest rate was 36° C.

The mean reciprocals for the time to complete development plotted against temperature, were fitted to logistic curves.

*Acknowledgments.* The authors are indebted to Professor J. Davidson and Mr. H. G. Andrewartha for advice given in the carrying out of the experimental work, and in the preparation of the paper.

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# INTRACELLULAR OXIDASES IN INFECTION

by E. SINGER

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*(Accepted for publication 6th November, 1944.)*

In previous communications the detoxication of various bacterial toxins by metal-catalysts has been described (Petherick and Singer, 1943, 1944a). Iron is capable of producing this detoxication both *in vivo* and *in vitro* provided certain biological redox-substances, such as ascorbic acid and adrenalin, or hydrogen peroxide are present as activators.

From the fact that ferrous sulphate dissolved in serum is more active than the same compound in saline or broth, and from the high activity of cytochrome *c*, it has been deduced that the biological detoxication of toxins is part of the function of the iron-containing enzymes in organ-cells of the living animal.

Investigations designed to assess the changes in the activity and distribution of these enzymes during infection or toxaemia were prompted by two facts which, although known for a long time and regarded more or less as curiosities, are of significance when considered with our recent observations.

It has been known for at least 30 years that the oxidase-reaction which can be obtained by various histochemical methods in the granules of the polymorphonuclear leucocytes disappears in severe infectious diseases. Further, the activity of iron-and-copper-containing enzymes decreases in predictable fashion during reaction with the substrate.

The importance and the theoretical implications of the results which can be obtained in studying biological oxidations by histo-chemical methods cannot be discussed here. The facts are summarized in the papers of Sehrt (1929) and Loele (1931).

The object of the present investigations was to study changes in the cells of various organs which are demonstrable in preparations stained with the so-called oxidase stains. These methods stain the oxidation points which are considered to be identical with the location of the oxidative enzymes, mainly the cytochrome-cytochome-oxidase system. Histological changes which can be observed using ordinary stains will not be described.

## MATERIAL.

The following material was employed: eighteen guinea-pigs of approximately 300 gm. weight either infected with virulent diphtheria bacilli or injected with a fatal dose of diphtheria toxin; 11 normal animals as controls; 16 mice injected with a fatal dose of purified *Cl. welchii* D toxin; 9 normal mice as controls; 4 hearts of young human adults: 2 from patients with chronic septicemic conditions, one endocarditis lenta, one chronic thrombophlebitis after appendicectomy, 2 from patients dying suddenly from non-infective diseases, one glioma, one status epilepticus. The human material was obtained through the courtesy of Dr. Willis, Pathologist to this Hospital.

## PREPARATION OF MATERIAL.

The animals were killed 2 to 6 days after injection of toxin, or after infection. The animals were bled, the organs removed and washed with saline. Frozen sections, 5 to 10$\mu$ thick were prepared immediately, spread out on saline solution, placed on a slide and left to dry in the incubator. Staining was performed as soon as the sections became fixed to the slide, usually 3 to 4 hours after preparation. Teased preparation of heart muscle were prepared and stained immediately. The human hearts were obtained 4 to 12 hours after death.

For work with fixed material, small pieces of organ were fixed in neutral formol saline for 16 hours, sectioned and stained. Blood films were fixed for 30 seconds with formol-alcohol and counterstained with haemalum or fixed in 50 p.c. alcohol for 2 minutes and counterstained with watery Giemsa solution.

The intensity of the oxidase reaction is somewhat lessened by fixation with formalin, but otherwise no essential differences were noted. Material which has been in contact with formalin solution for more than a few days is unsuitable. Blood films should be stained within a few hours after preparation.

Preparations of infected or of toxaemic animals were always stained in the same staining solutions with control preparations from normal animals which had been killed at the same time as the experimental animals.

## METHOD OF STAINING.

*General remarks.* True "oxidase" stains, that is stains which rely upon the production of colour by enzymatic oxidation with molecular oxygen as oxidant, and "peroxidase" stains in which the oxidant is hydrogen peroxide, were used. The true oxidase stains, such as indophenol-blue stain and leuco-acid-fuchsin stain, when used without addition of hydrogen peroxide give better pictures in formalin-fixed material than the peroxidase stains, as only true cellular oxidases are stained by them. Staining methods which use hydrogen peroxide suffer from the drawback that the erythrocytes give intense oxidase reaction and therefore often obscure the histological picture. With the true oxidase stains the red cells remain unstained or give only a very faint reaction, which does not interfere with the study of the preparations.

The quality of the chemicals used for staining is more important than the actual method. The description of staining technique applies therefore only to the dyes which were used in these investigations, and considerable experimentation will be necessary if different material and reagents are to be employed.

*Indophenol-blue stain* (Schultze, 1917): α Naphthol Merck, dimethyl–paraphenylene–diamine kindly supplied by Prof. Davies, Chemical Department, Melbourne University.

Prepare 1 p.c. solution of α naphthol in saline by addition of minimal amount of strong NaOH. This solution should have an approximate pH of 10.

Prepare 1 p.c. solution of dimethyl–paraphenylene–diamine in saline and acidify with HCl to an approximate pH of 2 to 3. Equal parts of these solutions when mixed should give a solution of pH 7·5 which is most satisfactory for staining. The stock-solutions keep for one to two weeks. Prepare mixture immediately before use; filter, and stain sections on a slide or in a shallow dish. For the staining of blood films or heart-preparations, weaker solutions are preferable, prepared by diluting the stock solutions before mixing 1/5 to 1/20 with saline.

When successful, this preparation gives brilliant staining and excellent differentiation, but it is entirely unreliable with a tendency to overstaining and precipitations. The stain is unstable within a few hours. Stability can be increased by placing stained preparations in 1/20 diluted Lugol's solution for one to two hours.

*Benzidine–hydrogen peroxide* (Graham, 1918): Prepare stock-solution of benzidine in 50 p.c. alcohol. Immediately before use, filter and add 0·2 ml. of 3 p.c. hydrogen peroxide in 50 p.c. alcohol to 10 ml. of benzidine solution. Solution should have a pH of 6·5. Two samples of benzidine B.D.H. and two locally produced samples were tested. One sample B.D.H. and one locally produced sample were satisfactory. The stain is fairly reliable and stable for several days. Good staining of human organs and blood-films can be obtained and also of heart-preparations of guinea-pigs and mice. The depth of staining is usually unsatisfactory with liver, kidney and blood films of animals. This method is not recommended for differential counts for oxidase-positive and oxidase-negative leucocytes in the blood of animals.

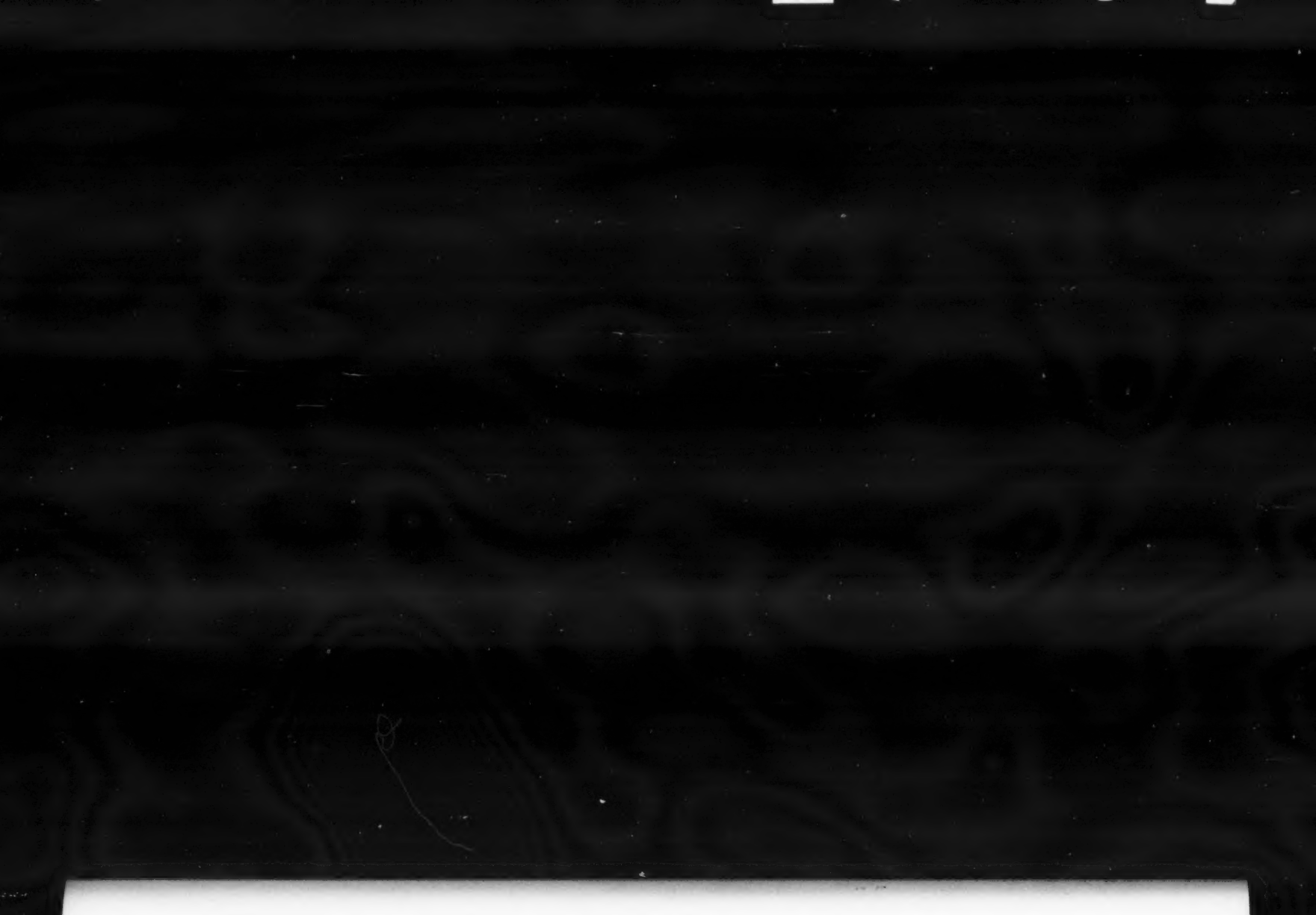
*Leuco–acid–fuchsin* (Lison, 1931): This method was used with slight modifications. Reaction of staining solution was adjusted to pH 6·0. Good results can be obtained when the stain is used as an "oxidase-stain" without addition of hydrogen peroxide.

Prepare leuco–acid–fuchsin and use within 2 or 3 days. Filter immediately before use. Stain for 10 to 30 minutes when hydrogen peroxide has been added, but for 3 to 6 hours without hydrogen peroxide.

This stain is fairly reliable, gives very good differentiation and is stable. The intensity of staining is too weak for use on blood films.

*Scarlet-red stain* (Goldman, 1929): Prepare stock-solutions of saturated sudan III or scarlet-red in 50 p.c. alcohol and in absolute acetone. Mix as required and filter immediately before use. The quality of the dye is decisive. Seven samples (4 Grueblers, 1 Merck, 1 Gurr and 1 scarlet-red, medicinal, of unknown origin) were tested. One sudan III Gruebler and the scarlet-red, medicinal, were found satisfactory. Stain for 3 to 6 hours, differentiate in 50 p.c. alcohol.

Careful comparison with the true oxidase stains gives complete parallelism of results in blood films and heart preparations. In sections from other organs fatty degeneration can become confusing for the inexperienced. This method gives brilliant staining, is reliable and the dye stable. It is the best method for the study of blood films.



## EXPERIMENTAL.

No essential differences were observed between human and animal material or between fresh and formalin-fixed preparations. The same changes were noted in organs of guinea-pigs infected with diphtheria bacilli or injected with diphtheria toxin as was the case in the corresponding organs of mice injected with toxin of *Cl. welchii* D. A general description is therefore given in the following paragraphs.

*Blood.* The loss of the oxidase reaction in the granules of the polymorphonuclear leucocytes during severe infections in humans is well known. Experimental investigations concerning the details and causes of this phenomenon have apparently not been performed. That this change is caused by the toxaemia and not by other factors accompanying the infection is shown in Table 1 in which the qualitative and quantitative changes in the white blood cells of guinea-pigs and mice are shown. All films were stained with indophenol-blue stain as well as scarlet-red stain, and counterstained either with haemalum or watery Giemsa solution. Control films were stained with the usual Leishman-Giemsa technique. Two hundred cells each were counted for the differential count.

### TABLE 1.

*Quantitative and qualitative changes in white cells of animals after injection of fatal doses of toxins.*

Guinea-pigs: diphtheria toxin.

| Time of count | Before injection. | 1 day after injection. | 2 days after injection. | 3 days after injection. | 4 days after injection. |
|---|---|---|---|---|---|
| Total number of white cells | 5,640 | 12,540 | 8,620 | dead | — |
| | 6,300 | 8,340 | 8,060 | 10,800 | dead |
| Number of polynuclear leucocytes | 3,380 | 6,640 | 4,480 | — | — |
| | 4,250 | 3,500 | 3,150 | 5,720 | — |
| P.c. fully granulated | 100 | 80 | 63 | — | — |
| | 100 | 61 | 59 | 53 | — |
| P.c. partly granulated | 0 | 20 | 32 | — | — |
| | 0 | 39 | 31 | 20 | — |
| P.c. ungranulated | 0 | 0 | 5 | — | — |
| | 0 | 0 | 10 | 17 | — |

Mice: toxin of *Cl. welchii* D.

| Time of count | Before injection. | 1 day after injection. | 2 days after injection. | 3 days after injection. | 4 days after injection. | 5 days after injection. |
|---|---|---|---|---|---|---|
| Total number of white cells | 20,280 | 20,940 | dead | — | — | — |
| | 15,240 | 19,200 | 20,160 | 20,100 | 16,500 | dead |
| Number of polynuclear leucocytes | 7,505 | 6,060 | — | — | — | — |
| | 5,330 | 6,330 | 10,890 | 8,640 | 6,030 | — |
| P.c. fully granulated | 100 | 78 | — | — | — | — |
| | 100 | 80 | 68 | 60 | 68 | — |
| P.c. partly granulated | 0 | 20 | — | — | — | — |
| | 0 | 20 | 22 | 28 | 22 | — |
| P.c. ungranulated | 0 | 2 | — | — | — | — |
| | 0 | 0 | 10 | 12 | 10 | — |

The oxidase-positive granulation in the leucocytes of man and mice is much more intense and dense than that in the corresponding cells of guinea-pigs. The granules appear as slightly irregular spheres 0·1 to 1$\mu$ in diameter. These fill the normal leucocytes of man and mice completely so that the nucleus is hardly visible. The limits of individual granules are visible only in the periphery of the cell. Guinea-pig leucocytes have 100 to 200 distinct granules, see Fig. 1. Nuclei and cytoplasm are not stained.

Severe changes in number and form of the granules are usually observed 2 to 3 days after infection or after an injection of toxin, see Fig. 2. The granules in a certain percentage of the cells disappear rather suddenly, in others the granules shrink and stain weakly, at the same time decreasing in numbers. It is quite common to find 10 to 50 p.c. of the cells without granules, or only partly granulated, together with cells which appear normal. The number of leucocytes increases after infection or in toxaemia. The nuclei of the partly granulated cells are strongly lobulated. The damaged cells are therefore apparently not juvenile cells released by the bone

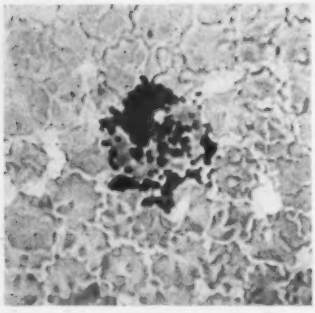


Fig. 1.

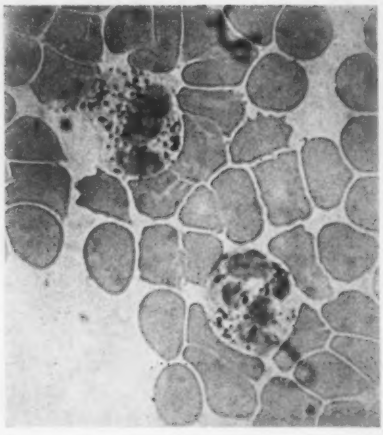


Fig. 2.

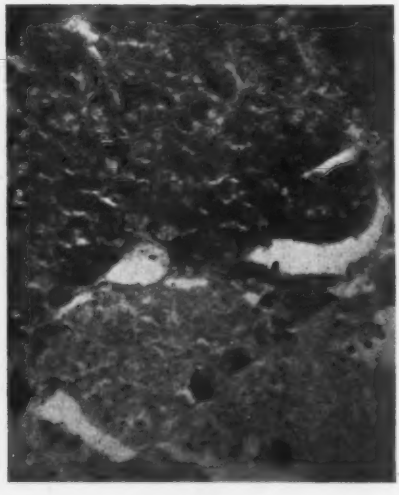


Fig. 3.

**Fig. 1.** Normal guinea pig. Polynuclear leucocyte. Scarlet-red haemalum. (× 1,000.)

**Fig. 2.** Two partially granulated polynuclear leucocytes from the same animal 3 days after injection of a fatal dose of diphtheria toxin. Scarlet-red haemalum. (× 1,000.)

**Fig. 3.** Normal guinea-pig. Adrenal. Indophenol-blue. Fixed in formol-saline. Deeply stained cortex above, unstained medulla below. (× 280.)

marrow under the stimulus of toxaemia, but those which have been in the circulating blood for some time.

*Heart.* The wall of the left ventricle was investigated in mice and guinea-pigs and the left papillary muscle in human material. Sections were cut preferably to show the muscle fibres in longitudinal aspect. All listed stains were applied. Control-sections were stained with ferricyanide–HCl but no positive reactions were obtained.

Muscle fibrils are faintly but clearly outlined. Most intensive staining however is found in the A discs which appear as cylindrical granules of uniform size surrounding single or a strand of fibrils. The Z discs appear as sharp deeply-stained lines, passing right through the fibres without showing the interruptions of the A discs. The I discs and intercalate discs remain unstained.

Scattered throughout the fibre and located between the fibrils and under the sarcolemma appears a variable number of small granules of slightly irregular size and form, usually more numerous in the vicinity of the nucleus. The nuclei are not stained.

In preparations from infected animals or man, and from toxaemic animals, the staining of the fibre is patchy and irregular in intensity. Often single A discs remain unstained and appear as clear gaps in an otherwise regular arrangement. The granules representing the A discs vary in size and form, become irregular and decrease in size. The staining of the fibrils suffers less than that of the A discs. The longitudinal striation is therefore accentuated to the detriment of the transverse striation. The number of the scattered granules increases, sometimes very considerably. No change can be observed in the Z discs or in the I or intercalate discs.

These changes appear one or two days after infection or intoxication of animals. Clear cut results, however, were obtained only in animals which survived for more than three to four days, or in human material.

*Liver.* The cytoplasm of the liver cells appears to be filled with fine granulations of slightly irregular size and form. The cells in the centre of the lobule usually contain fewer granules than those in the periphery. Nuclei of liver cells appear colourless and contain usually one to two deeply stained granules (nucleoli). The nuclear membrane appears to be stained but this cannot be ascertained with accuracy because of the intense oxidase-reaction of the granules in the cytoplasm of the cell. The intensity of staining in individual cells varies insofar as frequently single cells in a deeply stained strand of cells appear very much lighter or even unstained. Most cells contain a few unstained spaces (granules or fat droplets). Some red cells in the capillaries give oxidase reaction with indophenol-blue and leuco-acid-fuchsin-stain. Such cells are only found in liver and kidney-preparations. A few darkly staining clumps of undetermined nature not demonstrable in haemalum eosin preparations are observed in the capillaries and bloodspaces, or their neighbourhood.

In toxaemic or infected animals, wide areas are found to be unstained or so weakly stained that the margin of the individual cells is indistinguishable in preparations in which no counterstain has been used. Cells or strands of cells with normal oxidase reaction can always be found in these areas. The weakly-staining centre of the lobule is much more marked even in areas where oxidase stains otherwise give a normal picture. Blood-spaces and capillaries are clearly outlined, as if impregnated with a substance of oxidase-character. Many erythrocytes in the capillaries and blood-spaces give weak to fairly strong oxidase reaction but apart from that the capillary wall is clearly marked. The number of darkly-staining clumps, which do not give prussian-blue reaction, increases markedly. The plasma in the larger veins gives a faint oxidase reaction. Apparently the oxidases are released from the liver cells and are being carried away by the venous blood.

*Kidney.* The epithelial elements give stronger oxidase reaction than the mesenchyma; the cortex, apart from the glomeruli, gives a stronger reaction than the medulla. The collecting tubules show the weakest reaction of all the epithelial elements. The strongest reaction is found in the proximal convoluted tubules in which the oxidase granules are arranged in columns perpendicular to the lumen. The brush borders are clearly stained but somewhat weaker than the granules at the base of the cell. The capillary loops and the capsule of the glomerulus give only a very weak oxidase reaction. The nuclei appear as clear unstained spaces but the nuclear membrane and the nucleoli give a positive reaction.

In toxaemic or infected animals the oxidase reaction in the proximal and distal convoluted tubules becomes markedly weaker and somewhat irregular. Otherwise no marked changes can be observed.

*Adrenal.* Only formalin fixed material could be used for sectioning, as the consistency of fresh adrenals is too soft to allow cutting. In free-hand razor sections of fresh material, the principal changes which will be described for the formol-fixed gland could however be seen without difficulty.

Brilliant oxidase stain of the cortex is observed especially in the zona fasciculata and reticularis. The zona glomerulosa gives usually a much less intensive reaction than the other zones of the cortex. The oxidase reaction is located in or at the periphery of the lipid droplets of the cortical cells, whereas the cytoplasm of the cortex cells gives only a weak reaction.

The elements of the adrenal medulla remain colourless apart from stray cortical cells which are scattered through the medulla of animals, see Fig. 3.

In infected or toxaemic animals the picture is often obscured by haemorrhage. Further, leakage of substances giving oxidase reaction apparently occurs from dilated blood-vessels. The intensity of the oxidase reaction in the cortex, especially in the zona fasciculata decreases considerably. The lipid droplets of medium size, which give the strongest oxidase reaction in the cortex of normal animals lose the oxidase reaction sometimes as soon as two days after a dose of toxin which kills the control animals after four to six days. At this time the adrenals are somewhat swollen and hyperaemic but do not show any other marked changes of the histological picture.

*Other organs.* Skeletal muscle, thyroid, spleen and brain were investigated in a number of animals but no marked changes could be observed.

## DISCUSSION.

The fact that marked changes both in the intensity and distribution of the oxidases can be observed in the leucocytes and organ cells of the infected animals or man and in toxaemic animals gives additional support to the theory of the oxidative detoxication of toxins which has been mentioned in the introduction. This does not imply that the toxins are substrates of the oxidative enzymes but that toxin destruction is considered to be coupled with the oxidation of normal substrates. Toxaemia apparently accelerates oxidation and thus inactivation of the oxidases.

The polymorphonuclear leucocytes, the fibres of the heart muscle and the adrenal cortex are affected earlier and more severely than other organs. This is well in accord with a wide variety of clinical and pathological observations.

The oxidative processes in the living tissue are extremely complex and at present only incompletely understood. No information is available on the mechanism or on the biological importance of the oxidative breakdown of substances of protein character which could serve as a precedent for the elucidation of the detoxication of bacterial toxins. In a previous paper strong circumstantial evidence was presented that the main point of attack lies in the amino-groups of the toxin-molecule (Petherick and Singer, 1944b).

One observation, however, invites discussion. The oxidases of all organ cells are apparently closely linked with substances which can be stained with lipid-soluble dyes. In this connection the observations concerning the importance of phospholipids for oxidation (Elliott and Libet, 1944), the detoxication of bacterial toxins by sterols (Eisler and Gottdenker, 1938), and the lecithinase character of the toxin of *Cl. welchii* (Nagler, 1939) assume increased importance.

## SUMMARY.

Histochemical methods suitable for the demonstration of the cellular oxidases have been applied to material from infected animals and man, and from toxaemic animals.

Pronounced deviations from the normal picture were observed in the polymorphonuclear leucocytes, heart muscle, adrenal and liver. No marked changes could be observed in skeletal muscle, spleen, thyroid and brain.

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# CHEMO-PATHOLOGY OF PNEUMONIA IN CATS

by E. R. TRETHEWIE

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It has been shown that deaminating enzyme appears in increased amounts in the blood of man in pneumonia (Trethewie, 1942). In this paper investigations are made on the lungs of cats suffering from broncho-pneumonia, a condition which has been general among them for some years in Melbourne. In addition to assay of deaminating enzyme, tests have been made to show the liberation of histamine and to indicate the pathway of liberation.

## METHOD.

*Perfusion of the Lung.*

Under nembutal anaesthesia (180–360 mg. intraperitoneally) the right lung was excised and portions removed for section. The left lung was then perfused with Tyrode through the pulmonary artery and in one series the lung was excised and ventilated at 12 per minute (positive pressure inflation) with the hilum dependent. In another series the left lung was left in the thorax, the aortic arch tied and a cannula placed in the left auricle. A T-piece allowed the collection of tracheal fluid without disturbing ventilation (Fig. 1). Fluid was collected after the perfusion fluid appeared clear for 10, 20, 30 minutes and then hourly for three more hours. The lung was weighed after perfusion.

*Biological Assay of Perfusate.*

(a) Histamine.

Portion of each sample was boiled immediately and assayed for histamine on the isolated jejunum of the guinea-pig in a 4 c.c. bath.

(b) Deaminating Enzyme.

Adenosine was added in a concentration of 10$\gamma$ per c.c. to portions of unboiled samples. Part was immediately boiled and the remainder incubated at 38° C. for forty minutes and then boiled. The mixture was assayed on the guinea-pig heart preparation (Drury, Lutwak-Mann and Solandt, 1938).

*Extent of Pulmonary Lesion.*

Apart from histological examination of the lung, which was made on the unperfused right lung, the macroscopic appearance was classified as slightly diseased when there were only speckled areas affected; moderately when larger patches comprising the major part of one lobe were involved; and severely when most of the lung was affected.

## EXPERIMENTAL.

*The Liberation of Histamine.*

*(a) The liberation of histamine from the intact perfused lung.* It is rare to obtain from cats a disease-free lung. However, in one experiment the lung appeared normal and histologically showed only minor changes. The cat weighed 2·8 kg. and was pregnant. The volume of successive samples of fluid obtained from the auricle were 18·5, 31·6, 37·5, 30·2 and 24·1 c.c. 64·6 c.c. of tracheal fluid were collected. No histamine appeared in the auricular fluid during the first hour of perfusion. A trace was found in the next sample (1: 40 millions). No histamine was found in the third or fourth hour samples. The tracheal fluid was free of histamine. This gives a total output of 0·9$\gamma$ of histamine for the perfusion (exp. 1, Table 1). After perfusion the lung weighed 33·4 gm. and contained 21$\gamma$ of histamine per gram. Histologically one or two alveoli of the right lung contained a little fluid and one or two cells. The remainder were clear. Some interalveolar septa were broken down. There was also a small lesion around one bronchus. Thus the lung was practically normal.

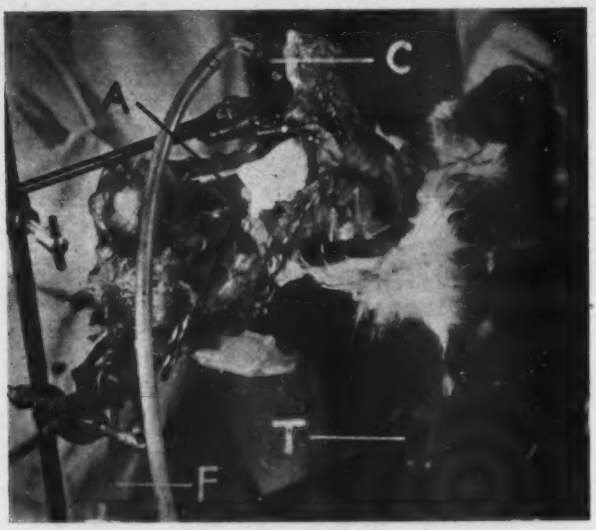


Fig. 1.

**Fig. 1.** Intact lung perfusion. A, Cannula connected to auricle. F, Drop of auricular fluid. C, Cannula in pulmonary artery. T, Tracheal fluid.

**Fig. 2.** Microscopic appearance of cat lung. Exp. 3. × 80. Details in text.

**Fig. 3.** Microscopic appearance of cat lung. Exp. 6. × 80. Details in text.

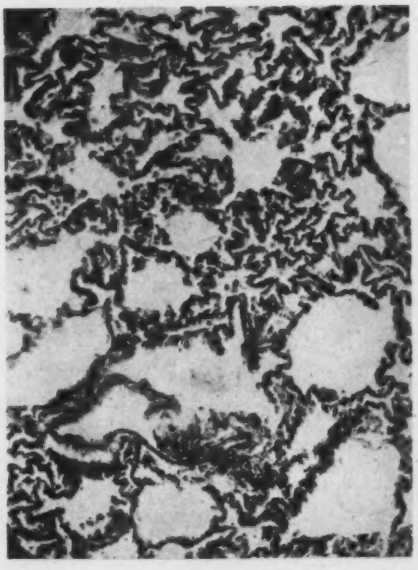

Fig. 2.

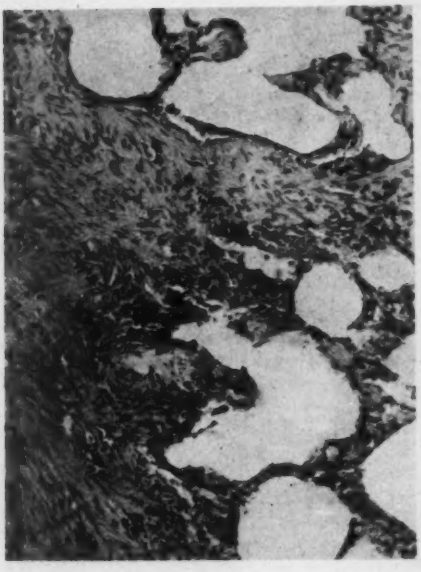

Fig. 3.

In four similar perfusions with lungs which appeared macroscopically slightly, moderately, slightly to moderately, and slightly to moderately diseased the following were the findings. In the first 6·6γ of histamine were detected in the auricular fluid and the lung contained 16γ histamine per gram after perfusion. The tracheal fluid was not tested. The second contained

2·2γ of histamine in the auricular fluid and 8·9γ of histamine in the tracheal fluid. The lung contained 6γ of histamine per gram after perfusion. The remaining two showed similar findings (exp. 4, 5, Table 1). It is thus seen that 6·6γ, 11·2γ, 9·6γ and 39·7γ of histamine were liberated from these lungs as compared with only 0·9γ of histamine from the normal lung.

TABLE 1.

| Cat No. | Appearance of lung. | Histamine content of lung. γ/gm. | Histamine output in fluid. Auricular. | Tracheal. |
|---|---|---|---|---|
| 1 | Healthy | 21 | 0·9γ | Nil |
| 2 | Slightly diseased | 16 | 6·6 | Not tested |
| 3 | Moderately diseased | 6 | 2·2 | 8·9γ |
| 4 | Slightly to moderately diseased | 25 | 1·8 | 7·8 |
| 5 | Slightly to moderately diseased | 50 | 4·5 | 35·0 |
| | | | Auricular, Tracheal and Lymphatic. | |
| 6 | Severely diseased | 70 | 12·6γ | |
| 7 | Slightly diseased | 20 | 22·6 | |
| 8 | Slightly to moderately diseased | 84 | 64·3 | |
| 9 | Moderately diseased | 17 | 23·0 | |

Histologically the first of these lungs (exp. 2, Table 1) showed numerous collections of small round cells around the bronchi and also around the vessels, congestion and collections of small round and other cells along the alveoli. Some groups of spindle-shaped cells were in the alveolar walls.

The second (exp. 3) showed collections of small round cells appearing fairly generally throughout. There were also some larger cells with more abundant protoplasm and an eccentric nucleus and macrophages. Occasional alveoli contained collections of fibrin with small round cells and other inflammatory cells. The vessels were normal. A portion of this lung is shown in Fig. 2 (× 80).

In the next (exp. 4) the alveoli contained groups of cells and fibrin.

The fourth (exp. 5) showed collections of macrophages and small cells along the bronchial walls and mucus in the bronchi. Groups of small round cells were seen and collections of fibrin.

*(b) The liberation of histamine from the excised perfused lung.* In the preceding experiments perfusion fluid was obtained from the auricle and the lymph vessels were distended. Tracheal fluid was also collected and this probably contained some fluid from the lymph vessels. It is possible by perfusing the lung with the hilum dependent and by opening the lymph channels to collect all the fluid from these regions. This was done and the findings are recorded in Table 1 (exps. 6–9). Histamine was readily detected in the perfusion fluid and the description of one experiment (exp. 8, Table 1) follows.

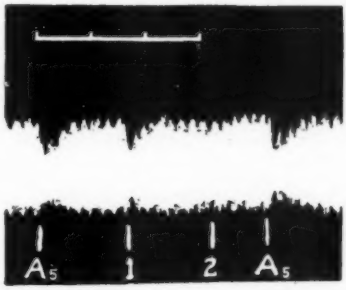


Fig. 4. Responses of the guinea-pig auricle to samples of perfusion fluid from exp. 9 to which adenosine has been added. At 1 response to 0·5 c.c. of sample boiled immediately. At 2 response following forty minutes incubation. Time in half minutes; details in text.

The lung of a pregnant cat weighing 4·5 kg. which contained small lesions was perfused. The volumes of the samples were 17·6, 17·4, 23·0, 56·5, 53·8 and 158·0 c.c. The histamine contents of the samples were 1: 16, 1: 6, 1: 1·6, 1: 3, 1: 3, and 1: 7 millions respectively. The lung weighed 20·1 gm. after perfusion and contained 84γ histamine per gm. The average output of histamine from these lungs was 30·6γ. The average output from the auricular fluid from the earlier group was 3·5γ and from the trachea 17·2γ.

Now the total output of histamine in γ per γ histamine content per gm. of lung averaged 0·9 in the case of the excised lung. In the case of perfusion collecting only tracheal and auricular fluid the corresponding figure was 0·5γ. With the almost healthy lung and similar perfusion it was 0·04.

Histologically the first of these lungs (exp. 6, Table 1) showed collections of small round cells and spindle shaped cells with some plasma cells and fibroblasts, some in small whorled groups replacing alveoli in areas. The vessels were not unduly evident and alveolar walls showed collections of small round cells and some thinning of the septa.

The second (exp. 7) showed some collections of small round cells and plasma cells and some larger groups of small round cells. Breaking down of inter-alveolar septa and thinning of the walls was noted.

The third of these lungs (exp. 8) showed some congestion. Collections of small round cells and plasma were evident. Some small round cells formed large groups. Breaking down of inter-alveolar septa and thinning of the walls was noted. Peribronchial accumulations of small round cells were evident. A portion of this lung is shown in Fig. 3 (× 80).

The fourth lung showed collections of small round cells in alveolar walls. Fibroblasts and small round cells were seen in some areas of older fibrous material.

*The Liberation of Deaminating Enzyme from the Lung.*

Samples of unboiled perfusion fluid were tested for the presence of deaminating enzyme. In general more deaminating enzyme was detected from perfusion of the excised lung than from the intact and this probably for the same reason as for the difference in the histamine since it was collected from more regions.

*(a) The excised perfused lung.* In one experiment (exp. 6, Table 1) the lung weighing 17·3 gm. was excised from a cat weighing 3·8 kilo and was perfused for four hours. The first and second samples were mixed as were the third and fourth and also the fifth and sixth. Adenosine was added in the proportion of 10γ per c.c. to each mixture and a portion boiled immediately. The samples were then found to contain 9γ, 10γ and 8γ adenosine per c.c. respectively. The remainder of the samples were incubated for forty minutes and then boiled. After incubation for forty minutes the contents were 7γ, 6γ and 5·5γ adenosine per c.c. The findings in the case of the third and fourth sample are shown in Fig. 4. At I, 0·5 c.c. of this mixed sample to which adenosine was added and immediately boiled is shown to equal 5γ adenosine ($A_5$). At 2, following incubation for forty minutes, the response to 0·5 c.c. of this sample was negligible. One c.c. of this sample had activity equal to 6γ adenosine. The fluid obtained was capable of breaking down 2·0 mg. of adenosine in forty minutes. A similar finding was obtained with experiment 8.

*(b) The intact perfused lung.* In an experiment in which auricular fluid was collected and the samples similarly tested the findings which were similar with similar perfusions were as follows. The cat weighed 3·5 kg. and the lung, which was slightly to moderately diseased, weighed 30·0 gm. after perfusion. In this experiment the auricular fluid was able to break down 0·63 mg. of adenosine in forty minutes. The tracheal fluid contained activity able to break down 0·53 mg. of adenosine in forty minutes. This is about half the activity developed in the perfusion fluid of the preceding experiment.

It is seen that the fluid when mixed with adenosine does not always show activity equal to the amount of adenosine added even when the samples are immediately boiled. The meaning of this is obscure. Possibly the fluid improves the contractility of the heart and so impairs the adenosine depression.

*(c) The normal lung.* In the experiment in which an almost normal lung was used the deaminating-enzyme activity of the perfusion fluid from the auricle and trachea was much less and there was no immediate depression of the activity of the added adenosine. The first and fourth samples showed no enzyme activity after incubation for forty minutes. The activity of all the auricular fluid was equal to breaking down 0·30 mg. of adenosine in forty minutes. Similarly the tracheal fluid was able to break down 0·16 mg. of adenosine. The difference in activity between this experiment and the preceding two types is greater than appears from these figures because in the first and second cases the immediate diminution in activity of the fluid is not included and incubation was then continued on a lower initial adenosine activity. The immediate diminution in activity was equivalent to 0·4 mg. of adenosine in the first experiment (excised lung) and 0·5 mg. in the second (intact lung) while in this experiment this activity was nil.

## DISCUSSION.

In this paper it has been shown that histamine is liberated from the perfused lung affected by broncho-pneumonia. This histamine appeared in small amounts only in the intra-vascular fluid. Larger amounts were detected in the tracheal fluid and when in addition the lymphatic vessels were opened much more was detected. It appears that most of the histamine passes into the lymphatic channels and possibly the trachea. Some qualification of these results is required since the lung was ventilated at a positive pressure and perfusion was with a saline. However, information derived from such perfusions is of value as has been shown in the case of acetylcholine (MacIntosh, 1938). If we may accept these findings the

probability is that in injury by pneumonia histamne passes into the tissue spaces rather than the blood vessels.

Earlier it was shown in human subjects (Trethewie, 1942) that deaminating enzyme appears in the blood in increased amounts when the patient has pneumonia. The content falls to a normal level with recovery. In this paper it is shown that some enzyme activity is found in the perfusion fluid obtained from a practically normal lung and that this is increased in the case of broncho-pneumonia. In addition in the case of perfusion fluid obtained from the diseased lung there is an immediate depression in the activity of added adenosine. This does not occur with the normal lung. The difference in enzyme activity of vascular fluid and lymphatic fluid is not so great as is the case with histamine. It would seem not unlikely that the increase in deaminating enzyme in the blood in pneumonia is due to liberation of the enzyme from the lung.

## SUMMARY.

Histamine has been shown to be liberated in broncho-pneumonia from the isolated, perfused and the intact, perfused lung of the cat.

Only a small amount of the histamine appears in the vessels, the majority passing into the lymphatic channels and the tracheal fluid.

Deaminating enzyme passes into the perfusion fluid from the normal lung of the cat.

There is an increase in the activity of deaminating enzyme in the perfusion fluid of the broncho-pneumonic lung, and also a substance is liberated which immediately impairs the cardiac depression produced by adenosine.

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# SALTANTS PRODUCED IN THE FUNGUS *CHAETOMIUM GLOBOSUM* BY MONOCHROMATIC ULTRA-VIOLET IRRADIATION AND A GROWTH EFFECT CHARACTERISTIC OF WAVELENGTH

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*(Accepted for publication 1st November, 1944.)*

Spores of *Chaetomium globosum* have been subjected to monochromatic ultra-violet irradiation and colonies have been grown from single spores which were irradiated dry. Special attention has been given to experiments in which the mercury lines at 265 mμ, 313 mμ and 334 mμ were used. In all these cases many saltants were produced as the result of the treatment of the spores. The primary object of the investigation was to observe any selective appearance of saltants at different wavelengths. Evidence for such a selective effect has been given by one of us, McAulay (1938) and the present experiments were made with more refined methods to obtain further information on this question.

A very marked selective effect was found in which a certain easily recognized type of change was produced in large numbers of colonies grown from spores irradiated by short wavelengths, but in a very few irradiated by long wavelengths. This change is more in the nature of a growth modification than of a saltant. A part of the colony is normal but a sector appears whose vertex usually is not at the centre of the circular growing colony. The sector is nearly clear of aerial mycelium and is frequently edged with dense brown in the substratum. The aerial mycelium is often dense at the edge of the sector and may form white knots. The modification has been designated by the letter "K."

There is a particular feature of interest about the K type. The colony, grown from a single spore, comprises distinct parts each with quite different characteristics. It would appear that the mycelium growing from the single spores is unstable in the sense that a colony derived from it may have two quite distinct forms.

On several occasions the same single spore gave rise to three or more types, normal, K and a saltant (Fig. 2, No. 6).

A very large number of saltant types is produced in *Chaetomium globosum* by irradiation with wavelengths over the range 230 mμ to 334 mμ. The percentage of colonies saltating is roughly constant over this range provided the energy per sq. cm. applied to the spore is a constant proportion of the lethal dose, in striking contrast to the "K" growth modification which appears selectively at short wavelengths.

## EXPERIMENTAL METHOD.

The monochromator used in these experiments was constructed in the laboratory, and has been described by McAulay and Taylor (1939). A 125 watt mercury discharge lamp, a commercial unit with outer envelope removed, was used as a source of ultra-volet light.

Spores were carefully spread on a coverslip in a narrow arc within the limits of the wavelengths and separated from one another so that all would have an equal opportunity of being irradiated. The spectrum was focussed on the spores by fluorescence of an underlying uranium

[1] The funds required for this work came from the Commonwealth Research Grant to the University.

glass mount; the control spores on another coverslip were placed near, but in the visible end of the spectrum. When the spores were treated at long wavelengths, short wavelengths were screened by means of glass microscope slides in order to eliminate the small amount of short wave radiation which was found to be present over the whole spectrum. One microscope slide was used for 313 mμ and three for 334 mμ. Even when these precautions were taken a certain amount of leakage occurred, and this is the possible explanation of a few K type appearing at the longer wavelengths.

An airstream was passed over the spores during irradiation to prevent the occurrence of secondary effects due to ozone accumulation, lack of aeration, high temperature and so on.

The spores were irradiated for varying lengths of time, for a few minutes at 265 mμ; a few hours at 313 mμ; and a few days at 334 mμ. Intensity measurements were made by means of a sensitive galvanometer and a vacuum thermopile. The average doses to give equivalent biological effects, exclusive of the K modification, were:

| | 265 mμ. | 313 mμ. | 334 mμ. |
|---|---|---|---|
| Average dose in joules/cm² | 0·1 | 25 | 150 |

Irradiated and control spores were plated singly in separate Petrie dishes so that the numbers of saltant colonies could be determined and each saltant obtained unassociated with mycelium from other spores.

Two techniques were used, the first a strictly controlled single spore method, and the second a dilution plate method. In the first dabs of spores were taken from the irradiated and control spore lines and placed in the centre of Petrie dishes on 1 p.c. clear malt agar. Single spores were picked up with a pointed platinum needle and placed at marked intervals on the agar. This plating was carried out under the high power of a dissecting microscope so that the spores, in their plated position, were easily visible and there was no possibility of more than one spore giving rise to the adult colony. All the leading features of mixed growth, etc., which are later to be described, were observed when this technique was used.

Spores remaining after the above plating were made into dilution plates, the amount of dilution depending on the percentage germination and visible colonies obtained from the plated single spores. When the colonies were visible to the naked eye they were cut out and placed in fresh dishes of malt agar, a single colony to a Petrie dish.

*Irradiation Dosage.*

In the earlier experiments 100 irradiated and 100 control spores were plated singly on 1 p.c. malt agar in Petri dishes, while in later experiments only 50 control spores were used, the larger number being considered unnecessary. The number of spores germinating was counted in this way, and also the number germinating and continuing to grow to form adult colonies. Percentage germination did not give a good indication of the effect of irradiation because in some experiments the control germination was very low. The percentage adult colonies from germinating spores gave a much better measure of the biological effectiveness of the dose.

## ANALYSIS OF SALTANTS.

Two strains of *Chaetomium globosum*, one, *Lj*, from Holland and the other, *KB*, from Sydney, and a globosum saltant, *Fld*, obtained in previous work, were used in these experiments. Fig. 1, No. 1, shows a normal colony of *C. globosum Fld*. A few additional experiments were performed with another saltant of *Chaetomium globosum* and with a strain of *Chaetomium elatum*, but owing to the difficulty of analysing the saltants of these two types they have not been included in the quantitative analysis (Table 1).

The changes which resulted from irradiating spores at 265 mμ, 313 mμ and 334 mμ were many and varied, but can be grouped into three rather broad classes, growth modifications, mycelial changes and perithecial changes. The first class contains those saltants or changes in which rate of growth or form is markedly different from that of the normal colony. Division of the growth types can be made into four sub-classes: photographs of typical examples of three of these are shown in Fig. 1, Nos. 2–4. The second class contains saltants in which the mycelium is very different from the normal. Here again division may be made into a number of sub-classes, and an example is shown in Fig 1, No. 5. Colonies in which the perithecia show a change in distribution, colour or size, make up the third class of saltants. An example is shown in Fig. 1, No. 6.

The mycelial and perithecial changes frequently appear together, and colonies are commonly found with marked growth modifications as well as perithecial and mycelial changes.

A point of interest is that saltants from irradiated spores often approach in appearance other species of *Chaetomium* (McAulay, 1938). Such resemblances may, however, be superficial only, no critical examination of material having been made.

Single irradiated spores would sometimes yield colonies in which a part was normal and a part saltant (Fig. 1, No. 6); less frequently, mixed saltants were obtained (Fig. 1, No. 7).

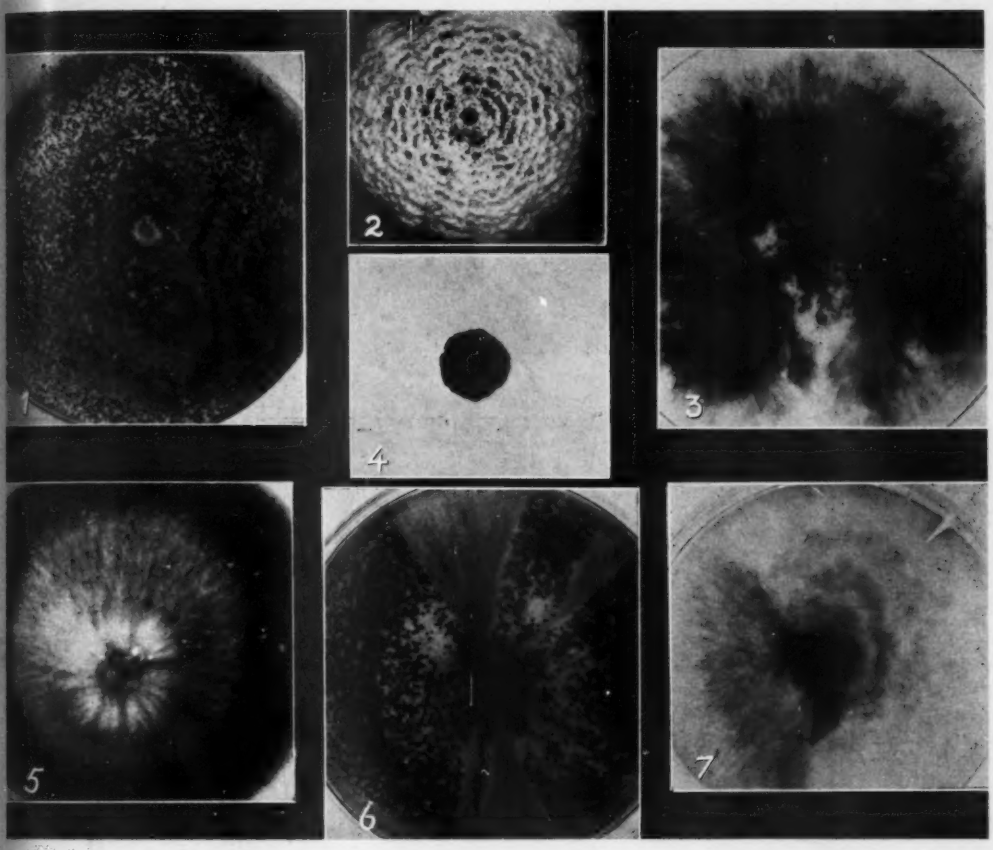


**Fig. 1.**

No. 1. *Chaetomium globosum* strain *Fld*: a normal colony grown from a single spore.

No. 2 Growth modification saltant resembling a succession of small scallops.

No. 3. Growth modification saltant with flares of mycelium growing from several central points, and with smaller flares further out giving the appearance of a red seaweed.

No. 4. Saltant colony which is small and button-like in form.

No. 5. Mycelial saltant with the central mycelium white and flat at first soon changing to fluffy; no perithecia. Saltant region surrounded by normal.

No. 6. Perithecial saltant sectors where mycelium is normal but perithecia are lacking: remainder normal. Colony grown from a single spore.

No. 7. A mixed colony grown from a single spore with two saltant types: scallop and perithecial saltant sectors.

All illustrations except No. 1 in Fig. 1 represent colonies of *Chaetomium globosum* grown from spores irradiated with monochromatic ultra-violet light. No. 1 has not been irradiated. Colonies are approximately three-quarters natural size.

### K-TYPE MODIFICATION.

Colonies from single spores showing the K type modification have characteristic areas, frequently sectors, in which growth is abnormal. Perithecia and aerial mycelium are not formed over most of the area, although they may be present at the edges. When the K-area is large the mycelium at its edge is compact and knotted, and the substratum is dense brown. A series of K-type colonies is shown in Fig. 2. Results obtained so far have shown that spore cultures from these colonies produce normal colonies while mycelium cultures produce either normal or K-type colonies. The fact that pure line spore cultures of K type cannot be made has prevented us from classing this modification as a saltant.

In Fig. 2 it will be noted that both normal and abnormal parts are present to a varying degree, indicating the instability of colonies from irradiated single spores. In Nos. 1 to 4 the normal and abnormal regions appear as sectors, the abnormal sectors arising very close to the centre of the colony and the normal originating at the centre.

It is seen from Table 1 that the K-type modification is produced commonly at 265 m$\mu$, a short wavelength, but rarely at long wavelengths. When production of the K-type is compared on the basis of equal lethal effect of the irradiation,

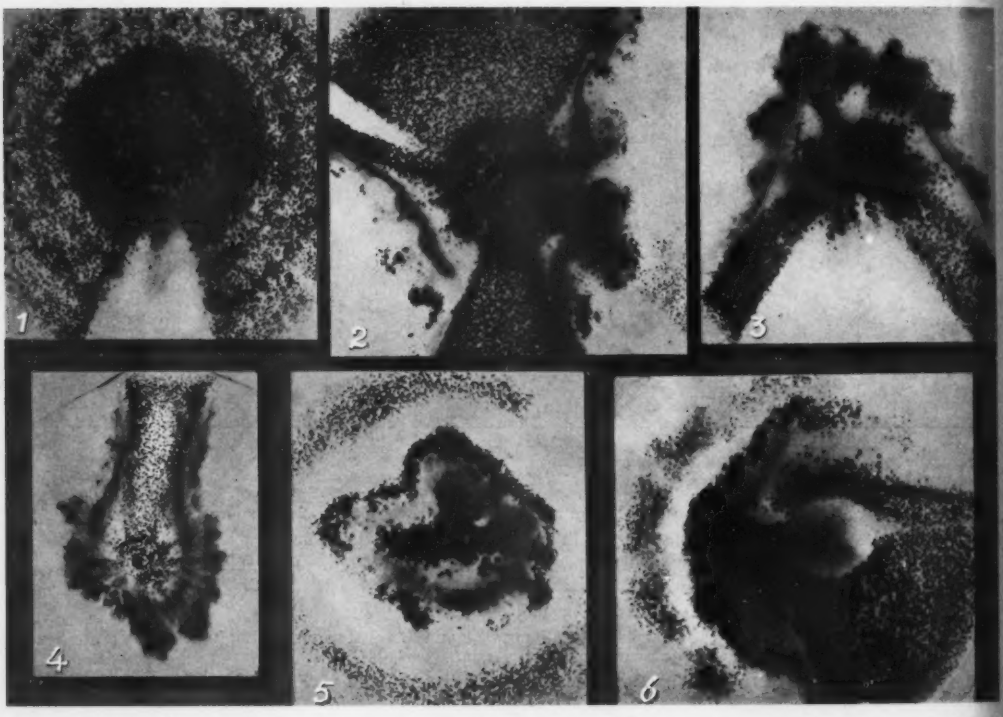


**Fig. 2.**

Nos. 1–6. Series of colonies of *Chaetomium globosum* showing the K-type modification characteristic of short wavelengths, ranging from a slight effect (No. 1) with little brown pigment, to a total effect (No. 5) with dense brown pigment round edge of affected area, an area devoid of aerial mycelium and perithecia. Growth normal only near limits of colony. No. 6 shows a mixture of 3 distinct types, normal, "K" and slow-growing saltant. Colonies represented in Nos. 4 and 6 are of single spore origin.

about 30 p.c. of the colonies growing from spores irradiated at 265 mμ show the modification, but less than 5 p.c. of the colonies when spores are irradiated at 313 mμ and 334 mμ (Table 1). The effect of irradiation is therefore selective in the production of K-type modification.

TABLE 1.

*Table of occurrence of saltants and a special growth effect, the K type, in colonies grown from irradiated and control single spores of* Chaetomium globosum Lj, Kb *and* Fld *(total of 41 experiments).*

| Wavelength | | 265 mμ. | | | | 313 mμ. | | | | 334 mμ. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Lj. | KB. | Fld. | Total. | Lj. | KB. | Fld. | Total. | Lj. | KB. | Fld. | Total. |
| Total experimental colonies | | 527 | 121 | 617 | 1,265 | 409 (293)* | 288 | 669 | 1,366 (1,250)* | — | 197 | 1,069 | 1,266 |
| Total control colonies | | | | | 700 | | | | 700 | | | | 650 |
| K type | exptl. | 126 | 58 | 212 | 396 | 61 (14)* | 3 | 20 | 84 (37)* | — | 0 | 17 | 17 |
| | control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 | 0 |
| Growth modification saltants | exptl. | 12 | 4 | 5 | 21 | 10 (5)* | 6 | 12 | 28 (23)* | — | 11 | 13 | 24 |
| | control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | — | 0 | 0 | 0 |
| Mycelial saltants | exptl. | 6 | 0 | 12 | 18 | 8 (5)* | 6 | 15 | 29 (26)* | — | 5 | 19 | 24 |
| | control | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | — | 0 | 0 | 0 |
| Perithecial saltants | exptl. | 2 | 3 | 5 | 10 | 5 (1)* | 0 | 1 | 6 (2)* | — | 0 | 8 | 8 |
| | control | 1 | 0 | 1 | 2 | 0 | 0 | 3 | 3 | — | 0 | 0 | 0 |
| Exptl. | percentage K type | | | | 31·30 | | | | 6·15 (2·96)* | | | | 1·34 |
| | percentage true saltants | | | | 3·87 | | | | 4·61 (4·08)* | | | | 4·42 |
| Average dose in joules/cm² | | | | | 0·1 | | | | 25 | | | | 150 |

* The figures in brackets omit an early 313 mμ experiment in which a large number of "K" type modifications appeared in contrast to all other 313 mμ and 334 mμ experiments. It is possible that contamination by 265 mμ occurred in this case.

## SUMMARY.

Further results are reported of effects produced by monochromatic ultra-violet irradiation of *Chaetomium* spores.

A refined technique is used enabling the effect of the irradiation on individual spores to be studied.

Saltations involving modifications of growth rate and form, mycelium and perithecia are produced, as well as a growth modification, the K-type.

Mycelium from the irradiated spore frequently shows instability, having the capacity for development into more than one type of colony. This property is particularly marked in the K type.

For equal lethal effects of the irradiation, production of K-type amounts to 31·3 p.c. at 265 mμ, but less than 5 p.c. at 313 mμ and 334 mμ, while production of saltants is independent of wavelength.

A selective effect with wavelength has been established for ultra-violet irradiation of biological material.

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# A CULTURAL REACTION FOR THE EARLY DIAGNOSIS OF *CLOSTRIDIUM OEDEMATIENS* INFECTION

by F. P. O. NAGLER[1]

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*(Accepted for publication 14th November, 1944.)*

In gas gangrene infections it is important to determine as rapidly as possible the infecting organism. Hitherto no satisfactory method for the rapid recognition of *Clostridium oedematiens* has been described. A special medium has now been devised for this purpose (Nagler, 1944). The principle feature of it is that it contains egg yolk and sheep blood. After only sixteen hours incubation *Cl. oedematiens* develop colonies which are quite characteristic and enable a diagnosis to be made.

## STRAINS AND MEDIA.

*Strains.*

A number of strains of *Cl. oedematiens* type A were obtained from the Council for Scientific and Industrial Research, Melbourne, through the courtesy of Dr. A. W. Turner and Miss C. E. Eales. They included the classical strains Weinberg, Jolly, Collins, Zeissler and Hall and four recently isolated strains from New Guinea. Strains of *Cl. oedematiens* type B and C and *Cl. haemolyticum* and nearly all other members of the genus *Clostridium* were obtained from the same source. Dr. Turner and the Commonwealth Serum Laboratories, Melbourne, kindly supplied antitoxins against *Cl. oedematiens* type A and B.

*Media.*

V.F. broth was used as the basic medium. This consists of a peptic digest of ox muscle and liver as described by Turner (1930) and later modified in his laboratory. Details of its preparation are given in an Appendix at the end of this article since the original paper is not easily accessible. 2·25 p.c. agar was added to the V.F broth. This was autoclaved for 20 min. at 15 lb. pressure, then filtered under low pressure through a thin layer of papier mâché in a Büchner funnel.

The egg yolk suspension was prepared by removing the yolk aseptically from the egg and placing it in a sterile Petri dish. The yolk membrane was then removed from the yolk and an equal volume of normal saline (approx. 15–20 c.c.) added and mixed thoroughly.

For making up the sheep blood agar plates containing the egg yolk, 1 part of egg yolk saline suspension, 1 part of defibrinated sheep blood and 8 parts of 2·25 p.c. V.F. were mixed after warming each of them separately to 45° C. After pouring, the plates were left open in the incubator to dry for five hours before inoculation.

The best fluid medium for quick growth of *Cl. oedematiens* was found to be a mixture of equal parts of saline liver extract (for detailed description see Appendix) and V.F. broth to which 0·05 p.c. of ascorbic acid had been added.

## THE CULTURAL REACTION.

All cultures were incubated in a McIntosh and Fildes jar which had been rendered anaerobic as quickly as possible after inoculation. Colonies which are irregular in shape develop to 0·8–1·5 mm. after 16 hours incubation on egg yolk blood plates inoculated from a rapidly growing fluid culture of *Cl. oedematiens*. When viewed by transmitted light the colonies are surrounded by an opaque haemolytic zone and outside that an almost circular dark red zone, which may be a reduction zone (Fig. 1).

The main diagnostic feature, however, is that the colonies as well as the haemolytic zone are covered by an opaque film with a pearl-like lustre (Fig. 2). When examined under the plate

[1] This work has been done under a research grant from the Commonwealth Government Scientific Liaison Bureau.

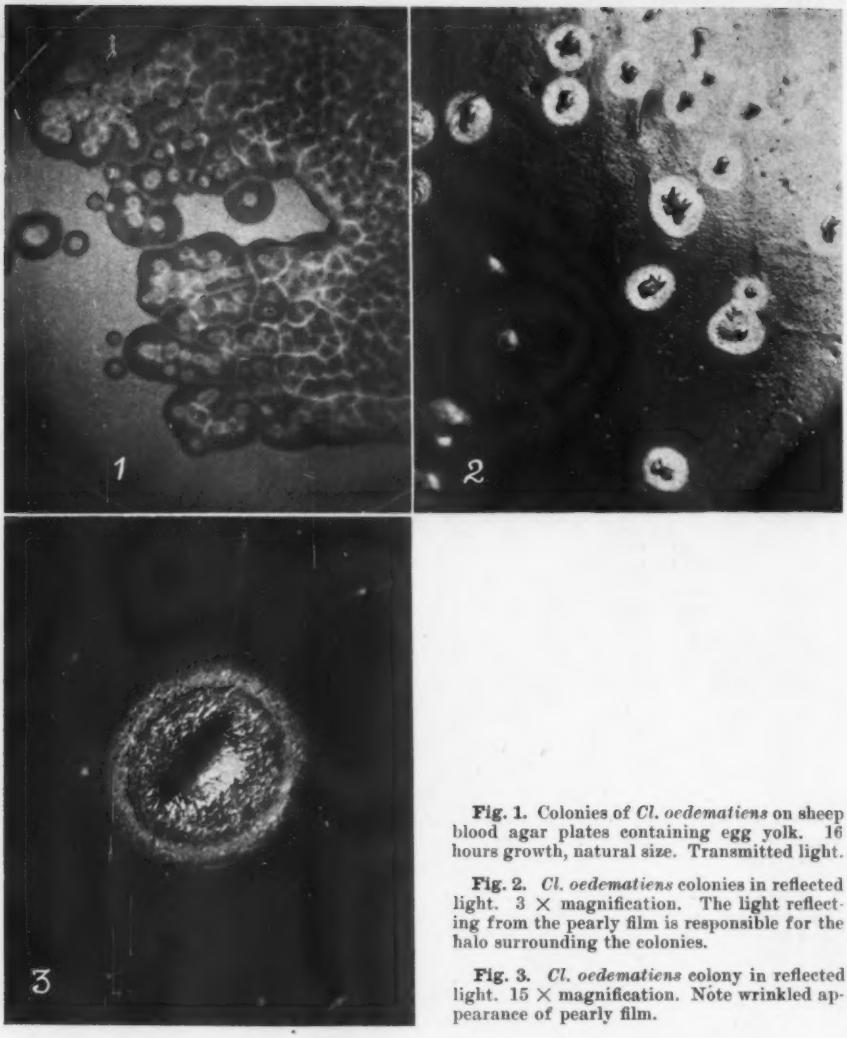


**Fig. 1.** Colonies of *Cl. oedematiens* on sheep blood agar plates containing egg yolk. 16 hours growth, natural size. Transmitted light.

**Fig. 2.** *Cl. oedematiens* colonies in reflected light. 3 × magnification. The light reflecting from the pearly film is responsible for the halo surrounding the colonies.

**Fig. 3.** *Cl. oedematiens* colony in reflected light. 15 × magnification. Note wrinkled appearance of pearly film.

microscope the surface of the film is seen to have short linear wrinkles distributed roughly in a concentric pattern (Fig. 3). No other anaerobic bacterium tested in this laboratory produced both the pearly film and the surrounding dark red zone.

*Cl. oedematiens* type B (black disease type and *B. gigas*) as well as two strains of *Cl. oedematiens* type C and *Cl. haemolyticum* did not give the pearl-like layer although they produced the dark red zone and in case of *Cl. haemolyticum* an extensive haemolytic zone.

*Cl. sporogenes, Cl. parasporogenes* and all 4 types of *Cl. botulinum* produced a similar pearly film on their colonies but did not produce a dark red zone.

A number of species of aerobic sporeformers of the genus Bacillus have been tested. None of them gave the pearly film.

Some strains of staphylococci have been found to give a very thin pearl-like lustre around their colonies. For further confirmation it was, therefore, made a routine to make smears of all suspicious colonies.

Samples of defibrinated blood from goats, rabbits and guinea-pigs were used in the plates and tested for the production of the dark red zones surrounding the colonies. The results with these bloods were as good as with sheep blood. Horse blood and human blood gave somewhat less satisfactory results even if used in a 5 p.c. concentration in the plates, because the outlines of the dark red zones are not as clearly defined as with the other bloods mentioned.

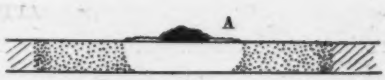


**Fig. 4.** Diagram of *Cl. oedematiens* colony with pearly film (A), haemolytic zone (B) and dark red zone (C) on sheep blood agar plate containing egg yolk.

## INVESTIGATION OF THE PHENOMENON.

So far it was only possible to carry out an incomplete investigation on the phenomenon of the production of the pearl-like film, which could be easily washed off the plate with water. Experiments were set up to determine the character of the agent which causes the production of this layer. It was found that antitoxins from two sources, when incorporated in the medium, did not inhibit production of either the pearly layer or the reduction zone. This contrasts with the way in which homologous antitoxin inhibits the appearance of a dark red zone around colonies of *Cl welchii* (type A) on sheep blood agar plates. Attempts were made in this laboratory to produce an antiserum against this agent by immunizing rabbits and guinea-pigs with whole cultures containing it. So far these attempts have failed.

Cultures of *Cl. oedematiens* in V.F. broth filtered in larger amounts (250 ml.) through a gradocol filter membrane A.P.D. 700 or a Seitz filter or freed from bacteria by high speed centrifugation still retained the capacity to produce a pearly film when spread over the surface of the plate. The film developed about 5 hours after the fluid was spread on the plate, which was kept at 37° C. and either exposed to the air or left under anaerobic conditions. If, however, smaller amounts of cultures containing that agent were filtered through Seitz filters or passed through gradocol membranes A.P.D. 700 the filtrates did not have this capacity.

Filtrates were stored for over 8 months in a frozen state at — 10° C. and were found to be still able to produce the pearly film. The production of the film was inhibited in the cold; plates inoculated with the filtrates and kept for 24 hours at 4° C. did not show the pearly film, but after removal of the plates to an incubator the film appeared in 5 hours. The agent is destroyed very quickly at 56° C. and at room temperature in 24 hours.

Titrations of filtrates using haemolysis (fowl, sheep and horse) and the specific reaction on egg yolk blood plates show a close parallelism which suggests that the agent may be identical with the haemolytic factor or factors.

Known enzyme-inhibitors, such as sodium citrate, sodium oxalate, hydrogen peroxide, amyl acetate, benzaldehyde, sodium taurocholate as well as trypsin, have shown no effect on the phenomenon when incorporated in the medium.

Egg yolk was extracted with peroxide-free ether, and two fractions thus prepared. When these fractions were added to sheep blood agar either separately or together the resulting medium showed only a very faint trace of the pearly film when inoculated with *Cl. oedematiens*. It is therefore possible that some substance in the egg yolk, perhaps a co-enzyme, was destroyed in course of the extraction.

Lecithin and cephalin were added in adequate amounts to sheep blood agar and the medium inoculated with *Cl. oedematiens*. There was no production of the pearly film on these media.

Although we have had the opportunity to test this method of diagnosis on actual clinical material in only five cases to date, our experience with these and with artificially mixed cultures suggests that it will be possible to detect *Cl. oedematiens* type A in material from lesions in which various other organisms are present.

*Acknowledgments.* I am greatly indebted to Dr. A. W. Turner of the C.S.I.R. for his helpful advice and interest in the work and also to Mr. N. Southern who prepared the photographs.

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## APPENDIX.

### METHOD OF PREPARATION OF THE V.F. CULTURE MEDIA AND LIVER EXTRACT

(As used in the laboratory of Dr. A. W. Turner).

The media are based upon a pig-stomach acid-digest of ox-muscle and ox-liver. In the following description, V.F. stands for "viande-foie", B. for "buffered".

*(a) Preparation of V.F. Acid Digest.*

The following constituents are taken in the following proportions:

| | |
|---|---|
| Lean ox-muscle | 100 gm. |
| Normal ox-liver | 100 gm. |
| Pig 's stomach | 120 gm. |
| Concentrated hydrochloric acid C.P. | 10 ml. |
| Tap water | 1,000 ml. |

The ox-muscle is prepared in the usual way, free from fat and aponeuroses; normal livers are selected and freed from large blood vessels, bile ducts, and other removable extraneous matter; and the pigs' stomachs are gently washed under running water and freed from scraps of fat, omentum, etc. Each is finely minced, mixed in the required amounts, and placed in 6 litre wide-mouthed bottles provided with rubber stoppers. The water is added, then the strong acid, the whole being stirred meanwhile. The bottles are stoppered and placed in a large water bath maintained at a constant temperature of 50° C. by means of some form of thermoregulator for 24 hours. At the end of this period a complete peptic digestion has occurred, and usually only a little sludge remains at the bottom of the jars. In order to inactivate the pepsin, the temperature of the water bath is thereupon raised to 80° C., and the digest filtered through moistened filter papers, placed in bottles, and heated again to 80° C. The product, which has a clear, light, yellowish-brown colour, is referred to as V.F. acid digest. Until needed, it is kept in a refrigerated room, where it remains usable for many months.

*(b) Preparation of B.V.F. Broth.*

Cold acid digest is filtered to ensure the removal of fat, and is then heated to 80° C.; sufficient of a 10 p.c. solution of sodium hydroxide (usually 40 to 50 ml. per litre) is added to raise the pH to 7·5 or 7·6 when tested at approximately incubator temperature in a Hellige comparator with phenol-red indicator. The mixture is steamed for 30 min. to facilitate the aggregation of the "earthy phosphate" precipitate, and then 1 p.c. of a mixture of buffer salts calculated for pH 7·4 is added and dissolved. It is then kept in the refrigerator overnight, in order to complete the aggregation of the precipitate. It is then filtered through paper, the pH is recorded (it should not be far removed from 7·4). The resulting broth is sterilized by filtration through a Seitz E.K. pad, and is tubed.

*Preparation of Saline Liver Extract.*

Fresh sheep liver is trimmed, skinned and minced. Normal saline solution is added in a proportion of 3 parts to 1 part of liver. The mixture is heated to 80° C. for 30 minutes, filtered through paper and sterilized by filtration through a Seitz E.K. pad and is tubed.

# STUDIES IN THE METABOLISM OF PLANT CELLS

## 3. THE EFFECTS OF CYANIDE ON THE ACCUMULATION OF POTASSIUM CHLORIDE AND ON RESPIRATION; THE NATURE OF THE SALT RESPIRATION.

by R. N. ROBERTSON AND J. S. TURNER

(From the Botany School, University of Sydney and the Botany School, University of Melbourne).

*(Accepted for publication 24th November, 1944.)*

The first two papers of this series (Robertson, 1941, 1944) dealt with the relationship of salt accumulation by carrot tissue to that part of the respiration induced by the application of the salt. It was suggested that the energy necessary for accumulation was derived from this "salt respiration".

It was shown by Lundegårdh and Burström (1933, 1935) that the accumulation of a salt in the roots of wheat plants was associated with the respiration induced by the salt (termed by Lundegårdh and Burström the "anion respiration"); this part of the respiration, unlike the ordinary or ground respiration, was inhibited by cyanide, and, with its inhibition, accumulation ceased. More recently the inhibitory effect of cyanide and the accumulation of ions has been confirmed by Hoagland and Broyer (1942) and its effect on both accumulation and respiration has been shown by Machlis (1944).

The purpose of this paper is to record and discuss the inhibition by cyanide of salt respiration and accumulation in carrot tissue.

The terminology used is that of previous papers of this series.

### MATERIAL AND METHODS.

The tissue used in these experiments was xylem parenchyma from carrot roots, cut into discs 0·8 cm. in diameter and 0·1 cm. in thickness. After being cut, the tissue was rinsed in several changes of distilled water and then aerated in distilled water until used for experiments. In the experiments here described the tissue was kept aerated for periods ranging from 115 hours to 165 hours to allow recovery of the respiration from the high rates which follow cutting.

The respiration rate was determined at 25° C. by measuring the oxygen uptake of approximately 1·3 gm. (25 discs) of tissue in Warburg vessels. At the beginning of each experiment the tissue was lightly blotted dry, weighed, and transferred to the Warburg vessels with 5 ml. of distilled water. After the steady respiratory drift in distilled water was reached, potassium chloride solution (0·3 ml.) was added from the side arm of the vessel. The concentration of the added potassium chloride was sufficient to give a 0·01 M. solution in contact with the tissue. In the controls a similar volume of distilled water was added from the side arm. The potassium cyanide was added when desired by stopping the experiment temporarily, removing the vessels from the manometers and adding the required amount of cyanide solution to the solution in contact with the tissue. The caustic potash solution in the centre tube of the vessel was then replaced with fresh caustic potash solution containing the appropriate quantity of potassium cyanide to give a vapour pressure of HCN in equilibrium with that in the solution (Krebs, 1935).

The salt accumulation was measured with replicate sets of tissue held in flasks in the thermostat and attached to the Warburg shaker to ensure aeration. A greater volume of tissue (50 discs) was used in these replicates than in the Warburg vessels but the same tissue/solution volume-ratio was maintained. The accumulation was followed by withdrawing samples of the external solution in conductivity pipettes and measuring the changes in conductivity. In addition, the final conductivities of the salt solutions in the Warburg vessels were taken and so the total salt absorption was obtained.

### RESULTS.

Five typical experiments will first be described *seriatim* and in the subsequent discussion the results common to the different experiments will be dealt with in detail. The respiration results

are plotted as the rate of oxygen uptake (in mm.$^3$/gm. F.W./hr.) against time and the accumulation results are plotted as the amount accumulated (gm. mol./gm. F.W.) against time.

*Experiment I.* (Fig. 1). In this experiment the salt respiration rate was the same at three different initial concentrations of potassium chloride, 0·005 M, 0·01 M, and 0·02 M. The different sets of tissue show slight differences in respiration rate in distilled water but show no drift in rate with time. Time from cutting: 165 hours.

*Experiment II.* (Fig. 2) shows the effects of salt and of potassium cyanide on the rate of respiration. After 80 minutes in distilled water the 0·01 M potassium chloride was supplied to the tissue and the rise in oxygen uptake was marked. The half-time of reaching the new steady state was short, as in the previous experiment. The drift in respiration rate after the addition of the salt runs parallel with that in the control, so the salt respiration remains constant. Two hundred minutes after the addition of salt, 0·001 M potassium cyanide was added to two of the sets and 0·0001 M to one; the fall in respiration rate was rapid. If we make the assumption, justified by later experiments, that the ground respiration of all sets drifts downwards in the same way, we can provisionally explain the cyanide effect as an inhibition of the salt respiration only. In this

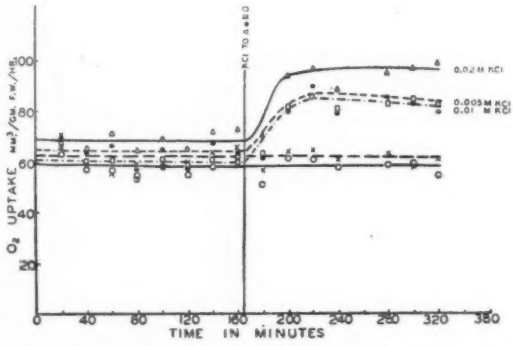



**Fig. 1.** The effect of three concentrations (0·005 M, 0·01 M and 0·02 M) of KCl upon the rate of oxygen uptake. Diacritic symbols: crosses and circles with white centres mark points for respiration rates in distilled water throughout the experiment; other symbols, initially in distilled water but with subsequent treatments indicated in the figure.

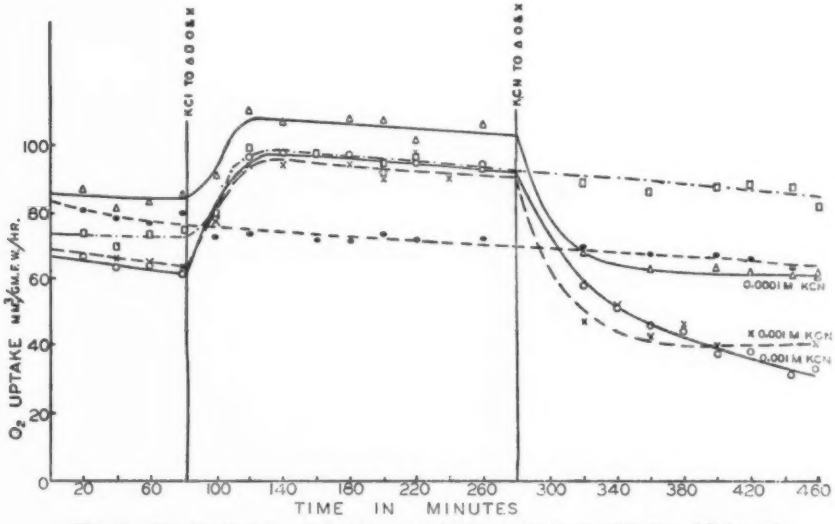
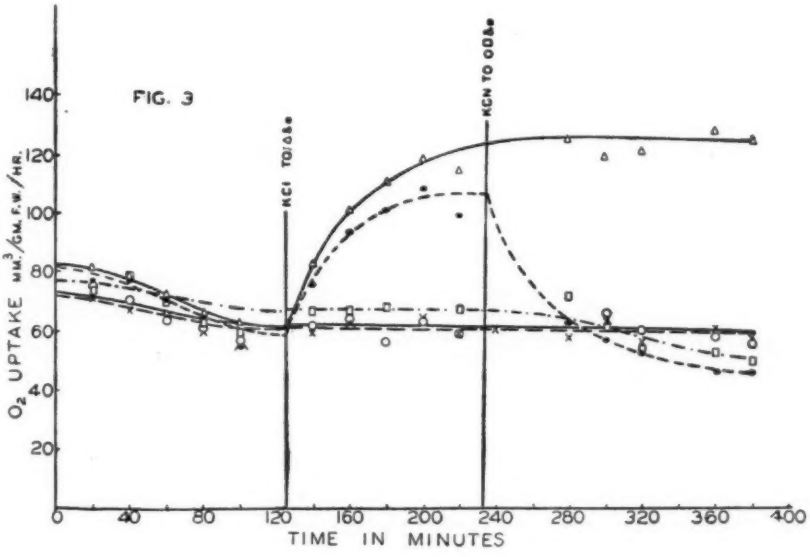
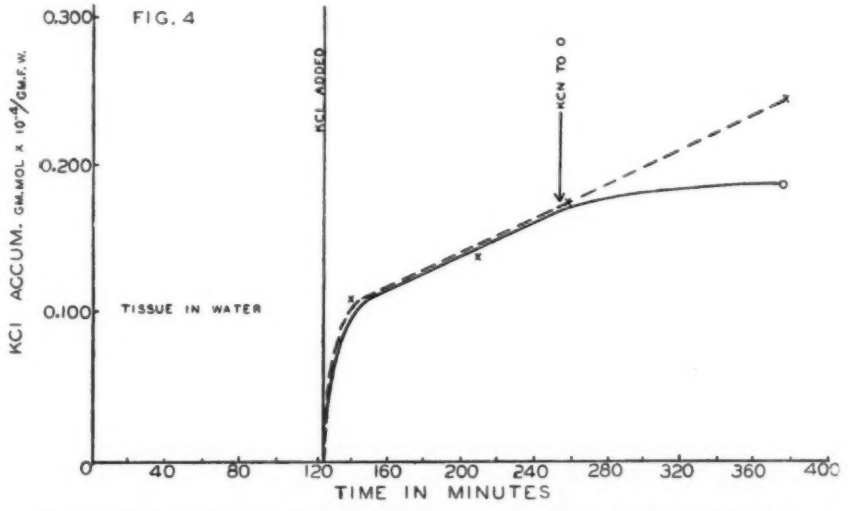



**Fig. 2.** The increase in respiration rate (salt respiration) with the addition of 0·01 M KCl; the decrease in salt respiration with the addition of KCN. Diacritic symbols: circles with black centres mark points for respiration rate in distilled water throughout the experiment; other symbols, initially in distilled water but with subsequent treatments indicated in the figure.

experiment the final conductivities were determined; these corresponded with an uptake of $0{\cdot}35 \times 10^{-4}$ gm. mol KCl/gm. of tissue in the set not treated with potassium cyanide and an uptake of $0{\cdot}28 \times 10^{-4}$ gm. mol KCl/gm. in that treated with 0·0001 M potassium cyanide. Time from cutting: 141 hours.

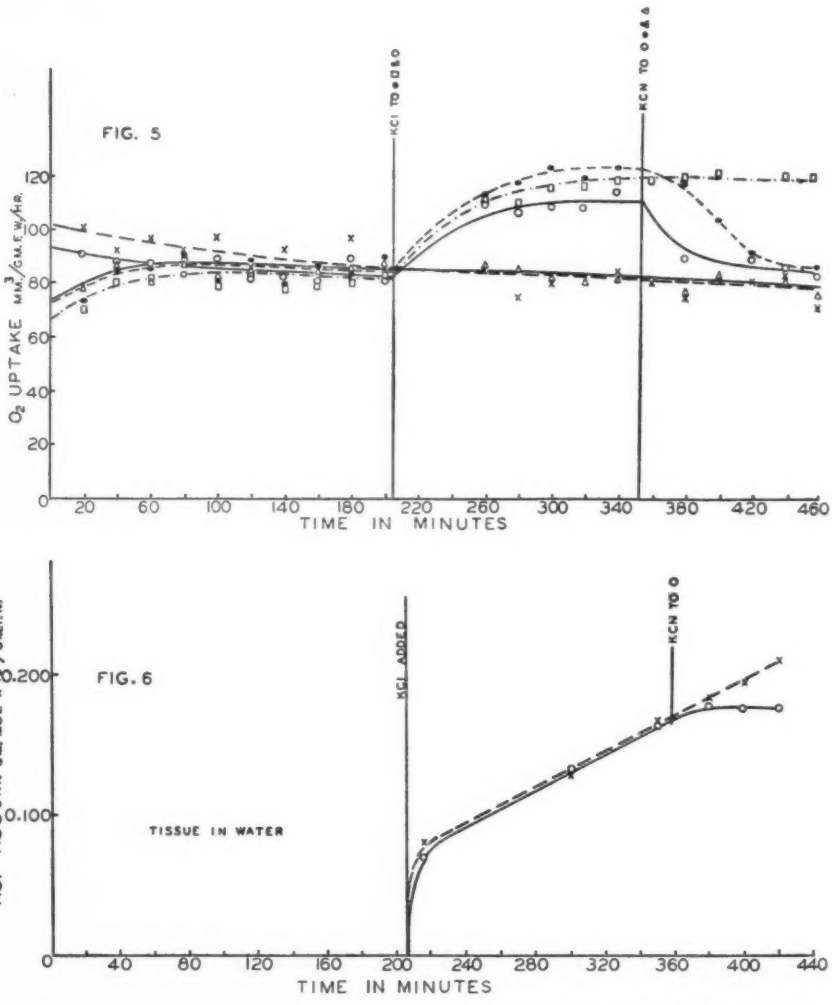



**Figs. 3 and 4.** The rate of respiration, the amount of salt accumulated and the effect of KCN on salt respiration, ground respiration and accumulation; replicate sets of tissue. Diacritic symbols: Fig. 3, crosses mark points for respiration rate in distilled water throughout the experiment; other symbols, initially in distilled water but with subsequent treatments indicated in the figure. Fig. 4, symbols as indicated in figure.

*Experiment III.* (Figs. 3 and 4). In this experiment a similar effect of potassium chloride on respiration is shown. Some time (110 min.) after the salt was added, potassium cyanide (0·001 M) was added to one of the sets in salt and also to two of the sets in distilled water. The salt respiration was completely inhibited, the total respiration returning to that of the distilled water controls, while the same concentration of potassium cyanide did not suppress the respiration in distilled water.

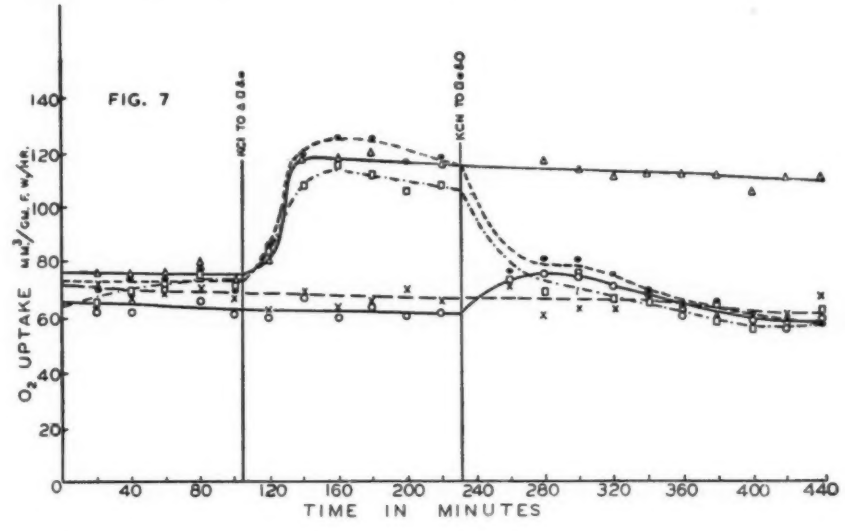
The conductivities of the solutions in contact with replicate sets of tissue were measured from time to time. From these measurements the amount of salt absorbed was calculated

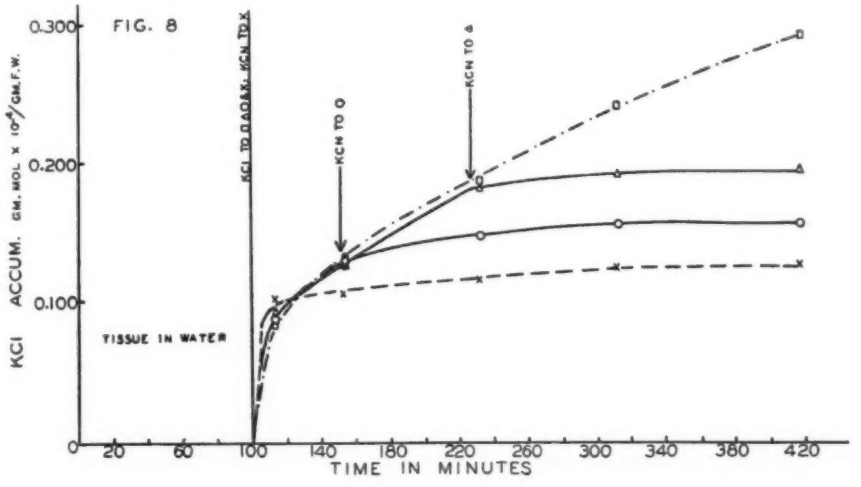



**Figs. 5 and 6.** The rate of respiration, amount of salt accumulated and the effect of KCN on salt respiration, ground respiration and accumulation; replicate sets of tissue. Diacritic symbols: Fig. 5, crosses mark points for respiration rate in distilled water throughout the experiment; other symbols, initially in distilled water but with subsequent treatments as indicated in the figure. Fig. 6, symbols as indicated in the figure.

and these amounts are shown in Fig. 4. After the salt had been in contact with the tissue for 126 minutes, 0·001 M potassium cyanide was added to one replicate. The amount accumulated after the addition of cyanide was very small: allowing for transitional effects, the graph indicates complete cessation of accumulation. Time from cutting: 163 hours.

*Experiment IV.* In Figs. 5 and 6 we plot the data for five sets of tissue, three of which received 0·01 M potassium chloride and two of which were left in distilled water. The rise in




**Figs. 7 and 8.** The rate of respiration, amount of salt accumulated and the effect of KCN on salt respiration, ground respiration and accumulation; the KCN was added to the accumulation replicates at different times after the addition of the KCl. Diacritic symbols: Fig. 7, crosses mark points for respiration rate in distilled water throughout the experiment; other symbols, initially in distilled water but with subsequent treatments as indicated in the figure. Fig. 8, symbols as indicated in the figure.

oxygen uptake in the presence of potassium chloride was again shown. After a further period, 0·001 M potassium cyanide was added to one of the sets in water and to two of the sets in potassium chloride. The salt respiration was inhibited but the distilled water respiration was unaffected by the potassium cyanide.

Potassium chloride, 0·01 M, was added to replicate sets when the potassium chloride in the Warburg vessels was tipped. The amount accumulated is shown in Fig. 6. Cyanide was added to one replicate and accumulation stopped. Time from cutting: 115 hours.

*Experiment V.* (Figs. 7 and 8), was a repetition of the previous experiment and gave similar results except that the control respiration showed an increase in rate of oxygen uptake immediately after the addition of inhibitor and the salt respiration was not inhibited so quickly (Fig. 7).

In this experiment four replicate sets of tissue were used and the inhibitor added to different sets at intervals. The amounts of salt absorbed (determined from the conductivity changes) show that the effect of adding cyanide is to inhibit rapidly the accumulatory mechanism (Fig. 8). If the cyanide is added with salt it does not interfere with the preliminary equilibration which occurs immediately after the addition of salt (Robertson, 1944). This is to be expected if this initial equilibration is a simple physical change not requiring energy expenditure by the cell. Time from cutting: 163 hours.

## DISCUSSION.

### *The Salt Respiration.*

These experiments confirm the results obtained in earlier work. After the addition of the potassium chloride the respiration rate rises rapidly to a new steady state. The total respiration (salt and ground) then follows, with time, a course approximately parallel to that of the control in distilled water; if this control respiration shows a fall with time, the total respiration also falls at approximately the same rate, the salt respiration remaining constant.

The half time of reaching the new steady state after the addition of the salt is about 20 minutes (see experiments I and II). This observation—that the salt increases the respiration so rapidly—is important in relation to explaining the nature of the process whereby the presence of a neutral salt (KCl) can initiate so marked a change in the cellular respiration. The average rates of ground respiration and salt respiration in the different experiments are compared in Table 1.

TABLE 1.

| | | $O_2$ uptake, mm.$^3$/gm. F.W./hr. | |
|---|---|---|---|
| Experiment. | Replicate. | Ground respiration. | Salt respiration. |
| I | 1 | 69 | 28 |
| | 2 | 60 | 25 |
| | 3 | 64 | 19 |
| II | 1 | 64 | 34 |
| | 2 | 63 | 37 |
| | 3 | 83 | 29 |
| | 4 | 71 | 28 |
| III | 1 | 72 | 59 |
| | 2 | 69 | 48 |
| IV | 1 | 86 | 34 |
| | 2 | 77 | 43 |
| | 3 | 81 | 42 |
| V | 1 | 63 | 43 |
| | 2 | 67 | 41 |
| | 3 | 72 | 50 |

There is no correlation shown here between the rate of ground respiration and the rate of salt respiration.

### *The Effect of Cyanide on Respiration.*

In previous work it was suggested (first by Lundegårdh) that the salt respiration might be a different kind of process from the ground respiration and be superimposed upon it; the effects of inhibitors on the two processes are therefore of considerable interest.

As can be seen in four of the experiments the addition of 0·001 M potassium cyanide results in a marked inhibition of the salt respiration bringing the total rate of oxygen uptake back to that of tissue in distilled water. Only in one experiment (experiment III) did the addition of cyanide have a depressant effect on the distilled water respiration and even there the maximum inhibition was about 25 p.c. In the other experiments, on treatment with cyanide, the respiration of tissues both in distilled water and in salt reach the same value, which differs little from that of the distilled water respiration.

The half-times of attaining the inhibited equilibrium vary from 44 minutes to 18 minutes. It is clear that the inhibition of the salt respiration by 0·001 M potassium cyanide is rapid and that the half-time is of the same order as that for the increase in respiration when potassium chloride is first applied. It is possible that both are controlled by the rate of movement of ions to the centres at which the respiration is taking place, though control by the rate of diffusion of oxygen to the cells must also be considered; the former possibility will be discussed subsequently when the uptake of ions is being considered.

These experiments confirm quite clearly the conclusions of Lundegårdh and Burström with wheat roots. The respiration induced by the presence of a neutral salt in the solution surrounding the tissue is sensitive to cyanide, thereby differing from the ground respiration which does not show the same sensitivity.

*The Absorption of Electrolyte.*

As pointed out in earlier work, an advantage of the conductivity technique for investigation of ion absorption is that it eliminates the complications due to ions from the external solution exchanging with ions from the tissue. The decrease of external conductivity, to a first approximation, represents the absorption by the tissue of both ions of the salt. It has been shown (Robertson, 1944) that immediately after the addition of salt to the tissue, there is a decrease in the external conductivity due to the entry of the salt as the result of the concentration differences between the external solution and the tissue. The same effect is apparent in these results (Figs. 4, 6, and 8). This can be proved by the following calculation: the initial concentration in the external solution (10 ml.) was 0·01 M potassium chloride; the volume of tissue was 2·4 ml. The dilution effect should therefore be 19·3 p.c., which would bring the external concentration to 0·00807 M. As seen in Fig. 8 the amount absorbed rapidly in the initial period can be taken as $0{\cdot}080 \times 10^{-4}$ gm. mol/gm. F.W. This is an absolute amount of $0{\cdot}192 \times 10^{-4}$ gm. mol and, since the volume of the external solution is 10 ml., it would reduce the concentration to 0·00808 M potassium chloride which agrees well with the calculated value.

Moreover, this equilibration occurs with a half-time of a few minutes and equality of concentration being attained rapidly, the process of accumulation begins. It has been suggested previously that the energy necessary for the accumulation is a fraction of that liberated in the salt respiration; the observation here recorded, that the accumulation begins early in the period of rising respiration, may be important for further investigation. In these short period experiments the accumulation curve approximates to the linear, though over longer periods (of days) it departs from linearity due to the increasing internal concentration. It is possible that the form of the curve in early stages of accumulation immediately after the initial equilibrium, may be the result of two opposing tendencies:

(a) to increase in rate because of increasing salt respiration, and
(b) to decrease in rate because of the increasing gradient against which the salt must be accumulated.

It may also be complicated, to some extent, by the exchange of ions which will occur and not be detected by the conductivity technique. If the ions leaving the cell differed markedly in their mobility from the $K^+$ and $Cl^-$ ions entering, some error would be introduced.

In any case, the rate of accumulation remained steady in each of the experiments where it was measured (experiments III, IV and V). The rate of accumulation and the average rate of respiration in these experiments are compared in Table 2.

TABLE 2.

| Experiment. | Mean salt respiration ($mm^3$ $O_2$/gm. F.W./hr.). | Accumulation rate (gm. mol KCl/gm. F.W./hr.). |
|---|---|---|
| III | 44 | $0{\cdot}34 \times 10^{-5}$ |
| IV | 43 | $0{\cdot}39 \times 10^{-5}$ |
| V | 45 | $0{\cdot}42 \times 10^{-6}$ |

*The Effect of Cyanide on Accumulation.*

The inhibitory effect of cyanide upon the process of salt accumulation is quite clear. Accumulation ceases a short time after the inhibitor is applied. The only exceptions to this are seen when the potassium cyanide was added with, or soon after, the potassium chloride in experiment V. There are two possible explanations of this slight absorption after the addition of the inhibitor: (a) it may be some adjustment other than accumulation proper; in this connection it should be remembered that if exchange were taking place and the ions entering the external solution from the cells had less mobility than the $K^+$ or $Cl^-$ ions, decrease in the external conductivity would take place, (b) the potassium cyanide might not have exerted its full inhibitory influence as quickly as in the other experiments. In this same experiment the time for the respiration of the replicates to reach the inhibited value was relatively long.

Whatever the explanation in this particular case it is clear that the cyanide inhibits completely the accumulatory mechanism. It is very important to note that it prevents the entry of further salt by accumulation but does not, at least during the period of the experiments, cause the loss of salt already accumulated. This suggests strongly that the mechanism responsible for the accumulation of salt is not the same as the mechanism which maintains the concentrations once accumulated. This agrees with other evidence discussed by Hoagland and Broyer (1942).

From the amount of salt absorbed immediately after its addition, it has been pointed out that the rapid movement of ions to equality of concentration within and without can be expected. The potassium cyanide may, therefore, be expected to enter the cells within a few minutes of its addition.

*The Relationship of Salt Respiration and Salt Accumulation.*

The contact of salt with the carrot tissue is sufficient to induce a type of respiration which differs from the ground or distilled water respiration in being sensitive to cyanide. As the salt respiration starts, accumulation begins. Both salt respiration and salt accumulation are stopped by the action of cyanide; these results are, therefore, strong evidence in support of the theory discussed in the earlier papers and first enunciated by Lundegårdh (1940) that the energy for the process of accumulation is liberated by the salt respiration. The inhibitory effect of cyanide on accumulation and on the total respiration in the presence of salt was confirmed by Hoagland and Broyer (1942) and again by Machlis (1944) though Hoagland and his collaborators have not recognized the existence of the component of the respiration here referred to as the salt respiration. It should be pointed out that

in his work with growing roots Lundegårdh (1940) found that the ground respiration and the respiration in distilled water were not quantitatively the same. This does not appear to be so with carrot tissue. Since the cyanide brings the respiration in salt back to that of control tissue in water and is usually without effect on the respiration of control tissue, the distilled water respiration and ground respiration seem to be quantitatively the same under the conditions of these experiments. It is necessary to emphasize that this remark should be restricted to tissue under these conditions because there is reason to believe that a cyanide sensitive respiration may be stimulated in ways other than by addition of salt (*cf.* discussion following, on work of Goddard and Marsh). Further, it is known that salts are liberated from carrot tissue after cutting and during the reabsorption period the tissue might be expected to show a cyanide-sensitive respiration. These experiments were, however, carried out with tissue washed for more than 100 hours after cutting.

## GENERAL DISCUSSION.

The results presented in this paper raise several questions of general biological interest as well as of particular interest in the problem of salt accumulation. These are

(1) The nature of the salt respiration,
(2) The nature of the ground respiration, and
(3) The mechanism of accumulation.

*The nature of the salt respiration.* It is now generally conceded (Green, 1941; Ball, 1942) that cyanide, inhibiting oxygen uptake, acts on cytochrome oxidase. Further, Marsh and Goddard (1939) using carrot tissue in Sörenson's M/60 phosphate buffer solution showed clearly that the respiration was inhibited by cyanide, carbon monoxide and azide, consistent with the behaviour of cytochrome oxidase. The light-reversible carbon monoxide effect disproved the possibility that catechol oxidase was the enzyme involved. The inhibition brought about by these substances (except at limiting concentration) ranged from 60 p.c. to 89 p.c., which in the light of our experiments suggests the possibility that the respiration investigated by Marsh and Goddard in buffer was a salt-stimulated respiration due to the ions present in the buffer entering the cell.

The results of Lundegårdh and Burström (1935) on wheat, of Machlis (1944) on barley and of this work on carrot suggest that the expenditure of energy in the process of accumulation can come about only if cytochrome oxidase is uninhibited. This linkage of a specific energy-consuming process (accumulation) to a specific respiratory process is of considerable interest since, except in the case of coupled phosphorylation reactions, so little is known of the energy utilization by organisms at the different steps in respiration. The insensitivity of the *Arbacia* ovum (unfertilized) to cyanide has been known for some time; the oxygen consumption increases upon fertilization and the additional respiration is sensitive to cyanide. Korr (1939) pointed out that both respiratory and functional activity in the fertilized *Arbacia* egg are inhibited by cyanide and that this might be a way of learning more about the utilization of the energy liberated in this process. Krahl and co-workers (1941) have shown that cytochrome oxidase occurs in the unfertilized ovum though cytochrome *c* cannot be isolated. Korr (1939) found that the respiration of the stimulated mammalian salivary gland was cyanide-sensitive though the resting respiration was not. Stannard (1939) showed that resting muscle respiration is sensitive to cyanide but not to azide. The stimulated muscle is however sensitive to azide, which brings the respiration down to that of the resting muscle. In this connection, too, the observations of Marsh and Goddard (1939) on mature and immature carrot leaves show another way in which the cyanide-sensitive respiration is modified. Respiration of young leaves is twice as active as that of mature leaves and is sensitive to cyanide while that of the mature leaves is not. Marsh and Goddard suggest the possibility "that two qualitatively different enzyme systems are operating in the young leaf, of which the cyanide-sensitive one is lost during development while the basic insensitive one persists". Commoner (1940) quotes many other examples of cyanide inhibition, among them Emerson's work on Chlorella and Genevois' work on Lathyrus. There seem, then, to be a number of physiological stimuli which affect the cytochrome system and which, in some cases, can quite rapidly start a component of respiration on which energy-consuming processes depend. Further discussion of this induced respiration raises the question of the nature of the ground (or resting) respiration.

*The nature of the ground respiration.* In the systems mentioned above two explanations have been advanced for the difference in the effect of inhibitors on the stimulated and unstimulated systems: (a) that the respiration in the unstimulated system proceeds by a mechanism different

from that of the stimulated system, thus "by-passing" the cytochrome, and (b) that the cytochrome system is operating in both but is sensitive to cyanide only when operating at higher rates.

Workers in the field of animal tissue respiration have been duly cautious in accepting, on the evidence of the action of inhibitors, the theory that some oxidation enzymes other than the cytochrome system operate in unstimulated tissues. There are two suggestions for the failure of cytochrome oxidase to show sensitivity to cyanide at lower activities. The first, due to Commoner (1940) suggests that at low rates the oxidase system is only partially saturated with substrate so that much oxidase can combine with HCN without noticeable effect on the remainder. The second, due to Ball (1942) suggests that the cytochrome oxidase, even in the presence of inhibitor, has an oxidation reduction potential which enables it to react normally with substances of low potential such as the flavo-proteins; on stimulation the cytochrome oxidase reacts with cytochromes *a*, *b* and *c* but the inhibitor lowers the potential sufficiently to make this reaction impossible.

The present results do not add any information which assists in deciding on the correctness of any of these views but are of considerable interest in showing that another stimulus—the addition of a neutral salt—can bring about the kind of respiratory change which has been observed elsewhere.

Lundegårdh and Burström (1935) indicated that the ground respiration in wheat roots, unlike the salt respiration, was catalysed by manganese. The possibility of a change from one enzyme to another must be considered and there are several ways in which it could be pictured as taking place after the addition of a salt to the cell. As suggested by Lundegårdh's observations (1940) a change of potential would have the necessary effect. A local redistribution of hydrogen ions is possible. In certain cases it seems probable that enzyme actions take place at liquid/liquid interfaces of the living membrane type and that the way in which a protein chain is spread at an interface may affect its properties as an enzyme. Bennet-Clark and Bexon (1943) draw attention to the peculiar labile nature of the enzyme systems of vacuolate plant cells, spatial disorganization of which results in almost complete loss of certain enzyme complexes. Conversely, spatial reorganization in the molecular sphere may initiate an enzyme reaction. It might be mentioned here that the salt effect is reversible (unpublished data), removal of the salt in contact with the tissue resulting in a slow return of the respiration rate to "ground" level. Finally, one of us (Turner, 1940) has stressed the necessity of taking account of the possible implications of the Pasteur effect in relation to ionic respiration. The increase in the rate of respiration of carrot tissue, brought about by wounding or by sodium nitrate, was accompanied by a decrease in the magnitude of the Pasteur effect.

*The mechanism of accumulation.* Lundegårdh (1940) suggested that the intensification of the oxidative system stimulated by the presence of salt could result in the accumulation of ions by the elimination of negative charges at the surface and the elimination of elementary positive charges at the accumulatory level. The possibility that cytochrome was specifically concerned in the mechanism was also suggested. It is significant that all mechanisms suggested by various workers as being thermodynamically possible depend on the continued evolution of ions by the cell.

Since many of the suggested mechanisms were published, Hoagland and Broyer (1942) have shown that the treatment of an accumulating organ with methylene blue results in an increase in respiratory activity but is without effect on the accumulatory mechanism. A similar observation on carrot tissue was made by Briggs and Robertson (Robertson, Ph.D. thesis, Cambridge, 1938) when it was found that the increased respiration due to methylene blue was not accompanied by accumulation. This suggests that the accumulation is not merely related to oxidation but is dependent upon a specific oxidation. It is not yet known what is responsible for the reduction of methylene blue in carrot tissue though it was shown by Turner (1940) that the dye increases triosis (glycolysis). It may be that the step in the respiratory process which brings about accumulation is the cytochrome oxidation stage; it is interesting in this connection that, whereas in the reduction of methylene blue two hydrogen atoms are taken up, in the cytochrome system a hydrogen ion is liberated into the system and an electron is transferred to the prosthetic group. The work of Machlis (1944), published while this paper was in preparation, is particularly interesting in narrowing the field of work on the connecting mechanism between respiration and accumulation. Machlis shows that in barley both respiration and accumulation are inhibited by malonate, which is generally recognized as a specific inhibitor for succinic dehydrogenase. It is well known that the cytochrome system is frequently reduced by hydrogens coming from an organic acid cycle and these observations, together with the inhibitory effects of cyanide, azide and iodoacetic acid, lead Machlis to conclude that the organic acid cycle is probably essential to salt accumulation. Preliminary experiments with malonate and carrot tissue do not indicate that succinic dehydrogenase operates (cf. also the work of Bennet-Clark and Bexon, 1943, with beet tissue). Machlis, like others in Hoagland's school, does not distinguish between "ground" and "salt" respiration. It is, however, significant that he found complete inhibition of accumulation when there was only 60 p.c. inhibition of the total respiration. Provisionally, then, we may interpret his results in our way. As stated earlier the salt respiration, though induced rapidly, disappears only slowly (over some 60 hours) when the tissue is restored to distilled water. Con-

sequently the total respiration may include ground respiration and residual salt respiration unless the tissue has been long enough in distilled water for the salt respiration to disappear. Machlis, however, used both a different tissue and a different technique from ours, and it would be premature to discuss his results more fully at this stage. We are of the opinion that a distinct salt respiration does exist in some tissues but the original suggestion of Lundegårdh—that it was an anion effect—cannot be taken as established on the evidence available.

The experiments described in this paper are considered important for two main reasons:

(1) They confirm Lundegårdh's theory of a cyanide-sensitive respiration which is induced by salt and on which accumulation depends,

(2) They give another example of the rapid initiation of a cyanide-sensitive respiratory system associated with living membrane activity—a phenomenon observed in other tissues under other kinds of stimuli.

## SUMMARY.

We have investigated the effects of cyanide on the respiration, the salt respiration and the accumulation of potassium chloride in cut carrot tissue.

It is shown that the salt respiration, which is induced rapidly (half-time of 20 minutes) after the addition of salt to the water surrounding the tissue, is inhibited by cyanide. The ground respiration shows no inhibition whether salt is present or not.

The initial absorption of electrolyte to equality of concentration inside and outside the cells is not prevented by cyanide; nor does cyanide cause the loss of electrolyte already accumulated. Cyanide completely inhibits, however, the process of accumulation against a concentration gradient. These results confirm Lundegårdh's theory, which was supported by the earlier papers in this series, that in at least some tissues the process of accumulation derives its energy from the salt respiration.

A process of general interest is discussed; that whereby a different kind of oxidation system (probably cytochrome oxidase) is rapidly induced by the application of a neutral salt to the tissue. The possibility that accumulation is dependent on a special energy-liberating process is considered.

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# PARTIAL AMINO-ACID COMPOSITIONS OF THE PROTEINS OF SOME LEGUME SEEDS: THE TYROSINE, TRYPTOPHANE, CYSTINE (PLUS CYSTEINE) AND METHIONINE CONTENTS

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It has been suggested by Lugg and Weller (1944), as a conclusion to be drawn from their analyses of various legume seeds and from the results of feeding trials by workers who have tested the nutritive values for rats and chicks of certain legume seeds and their proteins, that the methionine content of the seeds of any legume should be presumed low in the absence of actual experimental evidence to the contrary. Earlier, Lugg and Weller (1941) had shown that the whole protein of the embryos of ripe, dry seeds of *Lupinus angustifolius* were of rather low tryptophane and very low methionine content.

In certain districts of Australia, the small seeds of legumes such as *Trifolium subterraneum*, have developed an importance as stock (notably sheep) food during the dry summer period, just as have the large seeds of *Lupinus pilosus* sub. sp. *Cosentini* in parts of Western Australia. The seeds of *Trifolium subterraneum* are consumed in the fructification, or head, consisting usually of three or four one-seeded pods and the remains of numerous infertile flowers, whereas the *L. pilosus* pods are not normally eaten by the stock.

An opportunty has been taken of examining the seeds and the rest of the head material of *T. subterraneum*[2]. The tyrosine, tryptophane, cyst(e)ine (*i.e.* cystine plus cysteine calculated as cystine) and methionine contents of the "whole" seed protein were estimated. The sulphur distributions of the seeds and the cyst(e)ine and methionine contents of the protein in the heads minus seeds were also estimated, as were the tyrosine and tryptophane contents of the "whole" protein of the embryos of *Lupinus luteus*.

## EXPERIMENTAL.

### *The Preparations.*

The "whole" protein preparation from embryos of *Lupinus luteus* was the one made by Lugg and Weller (1944).

TABLE 1.

*Particulars concerning the original fructifications, or heads, of* Trifolium subterraneum.

Per 1,000 seeds (approx. 300 heads).

| Seeds. | | | Heads minus seeds. | | | Heads. | | |
|---|---|---|---|---|---|---|---|---|
| Fresh wt. gm. | Dry wt. gm. | P.c. N. | Fresh wt. gm. | Dry wt. gm. | P.c. N. | Fresh wt. gm. | Dry wt. gm. | P.c. N. |
| 7·31 | 6·93 | 5·80 | 10·92 | 9·71 | 1·64 | 18·23* | 16·64* | 3·38* |

* Values obtained by calculation from those shown in the other columns.

[1] On visit from the School of Agriculture, University of Sydney, N.S.W.

[2] The strain was "Dwalganup extra early". The sample was kindly provided by Mr. A. M. Stewart from material being used in feeding trials with sheep at the Institute of Agriculture, University of Western Australia, Nedlands, W.A.

The heads of *T. subterraneum* were allowed to dry off on the plants and were harvested in January, 1944. The dissection of the heads and the processing of the parts occurred some five months later. The seeds were separated from the rest of the head material, consisting of the pods and the remains of infertile flowers, and the relative amounts in which they occurred are shown together with the nitrogen contents in Table 1. "Whole" protein preparations were made from the powdered seeds and from the powdered heads minus seeds, by removing the non-coagulable and other non-protein nitrogenous materials and certain other substances, according to the procedure used by Lugg and Weller (1944). In Table 2 are shown the amounts of total N, coagulable N and protein N in the original materials, the percentages of N in the "whole" protein preparations, and other data concerning the preparations. Incidentally, the seeds contained considerable amounts of oil or fat. The seeds and heads minus seeds have been given the characteristic numbers 8 and 9 respectively, in direct continuation of the numbers used to characterize the preparations described by Lugg and Weller (1944).

TABLE 2.

*Preparation of "whole" protein samples from seeds and heads-minus-seeds of* Trifolium subterraneum.

| No. | Material. | Dry wt. gm. | P.c. N. | Total N mg. | Coagulable N mg. | Protein N mg. | Dry wt. protein prep. gm. | P.c. N protein prep. |
|---|---|---|---|---|---|---|---|---|
| 8 | Seeds | 6·93 | 5·80 | 402 | 326 | 325 | 4·23 | 7·69 |
| 9 | Heads minus seeds | 9·71 | 1·64 | 159 | 148 | 146 | 8·64 | 1·69 |

*Methods of Estimation.*

Nitrogen estimations were made by the Kjeldahl method.

Tyrosine and tryptophane contents were estimated by the methods described by Lugg (1937, 1938a), with observance of the precautions mentioned by Lugg and Weller (1941). The cyst(e)ine and methionine contents of the protein preparations and the distribution of sulphur in the seeds, were estimated by the differential oxidation procedure described by Lugg (1938b), the total S estimations being made by the procedure described by Lugg and Weller (1944).

RESULTS.

The amino-acid contents, as percentages of the nitrogen present occurring in these forms, are shown in Table 3. The sulphur distribution in the seed of *T. subterraneum* is not shown as such, but the cyst(e)ine and methionine contents calculated therefrom are shown together with the sulphate–S content. The sulphate–S contents have also been expressed in relation to the N present. The assumptions underlying these calculations are that the sulphur is present only as cyst(e)ine and methionine (as free amino-acids or as amino-acid residues in protein) and sulphate, and that the amino-acid residues behave with the reagents in the same ways as do the free amino-acids. Reference has been made by Lugg and Weller (1944) to the validity of these assumptions, and in accordance with their practice, the cyst(e)ine and methionine contents of the seeds have been shown in italics to indicate that they may be vitiated by inclusion of other sulphur-containing compounds, because, whereas the presence of sulphur in combinations other than those envisaged in the assumptions has not been demonstrated with certainty in proteins, many seeds are known to contain sulphur in other combinations.

TABLE 3.

*Partial compositions of the preparations: losses during extraction.*

| No.* | Preparation from | P.c. N. | P.c. Tyr. N. | P.c. Trypt. N. | P.c. Cyst. N. | P.c. Meth. N. | $\frac{SO_4}{N} \times 100$. | P.c. loss, or apparent loss, during extraction. Cyst. | Meth. | $SO_4$—S. |
|---|---|---|---|---|---|---|---|---|---|---|
| 4W | *Lupinus luteus* embryos (ripe) | 12·7 | 1·41 | 0·61 | 2·07† | 0·37† | 0·03† | | | |
| 8 | *Trifolium subterraneum* seeds (ripe) | 5·80 | | | *0·38* | *0·30* | 2·40 | 4 | 11 | 91 |
| 8W | " " | 7·69 | 1·56 | 0·89 | 0·45 | 0·33 | 0·27 | | | |
| 9W | *Trifolium subterraneum* Heads minus seeds. | 1·69 | | | 1·52 | 0·28 | 0·47 | | | |

* The suffix "W" signifies the "whole" protein preparation made from material bearing the characteristic number.

† Values previously reported (Lugg and Weller, 1944).

The values shown are the means of satisfactory replicate estimations. The random errors are such that the values are believed to be in error by not more than the following: 0·04 for tyrosine; 0·02 and 0·03 for tryptophane in 4W and 8W respectively; 0·02, 0·04, 0·02 and 0·05 for cyst(e)ine in 4W, 8, 8W and 9W respectively; 0·03 for methionine, and 0·01, 0·07, 0·03 and 0·08 for sulphate in 4W, 8, 8W and 9W respectively.

On the right of Table 3 are shown the apparent losses of cyst(e)ine and methionine and the loss of sulphate, during extraction in the preparation of 8W from 8. These losses have been calculated with the aid of the data shown in Table 2, and those for cyst(e)ine and methionine are qualified as "apparent" in view of the possibility, already discussed, of the vitiation of the cyst(e)ine and methionine values for 8.

It is considered that the random errors of estimation can easily account for the cyst(e)ine and methionine losses. Clearly, there could have been very little free cyst(e)ine and methionine in 8, and at the same time (apart from sulphate) other sulphur containing compounds could not have been present in more than small quantities.

## DISCUSSION.

This study, like the earlier work with legume seeds (Lugg and Weller, 1944), was concerned primarily with the nutritive values of the materials under consideration. The suspicion expressed by Lugg and Weller (1944) has been confirmed for the seeds of *T. subterraneum* and for the "whole" proteins in the seeds—they are of very low methionine content. They are somewhat unusual in being of very low cyst(e)ine content as well. Indeed, from the general nutritional standpoint (see Lugg and Weller, 1941, 1944), these seeds seem to be less well supplied with the sulphur-containing amino-acids than any of the other legume seeds that have been investigated by these methods. Rose (1937) considered that the requirement of methionine for normal growth of the rat might be stated tentatively as 0·6 p.c. of the food intake, and Grau and Almquist (1943) have estimated the methionine requirement for optimal growth of the chick to be about 0·5–0·6 p.c. of the food intake when plenty of cyst(e)ine is present. Thus, even when providing 20 p.c. of protein in the diet, the seeds of *T. subterraneum* could furnish only a small fraction of the estimated methionine requirements for satisfactory growth of rats and chicks. Little is known, however, of the dietary requirements of sulphur-containing amino-acids for sheep. Considerable cystine is required for wool growth, and although methionine might be regarded as a source of such cystine there is some uncertainty about this (Marston, 1939). The question whether amino-acids or protein can be synthesized in the rumen, is also involved.

The digestibilities of the proteins in the seeds are probably high for most animals, but the protein in the tough, fibrous heads minus seeds would probably be less accessible, even to ruminants. In any case, less than one-third of the protein in the heads we have examined, was contained in the heads minus seeds. Furthermore, the methionine content of the protein is about as low as that of the "whole" protein in the seeds. On the other hand the cyst(e)ine content is similar to that of the "whole" proteins in the leaves of land plants generally, and to the extent that it should happen to be accessible to the animal it could exercise some sparing action upon the small amount of methionine available. It seems unlikely that the small amount of non-protein N in the heads minus seeds would be represented at all largely by methionine. Incidentally, in reference to the rather unusual distribution of sulphur in this protein, it should be pointed out that during development the tissues of the heads are almost devoid of chlorophyl and must be almost lacking in photosynthetic function.

Additional support for the view that legume seeds and their proteins tend to be of low methionine content has been provided by the findings of Woods, Beeson and Bolin (1943) and Petersen, Lampman, Bolin and Stamberg (1944) that for rats and chicks respectively, methionine is the main growth-limiting deficiency of Alaska field peas (*Pisum sativum*). Lugg and Weller (1944) found 0·80 p.c. and 0·66 p.c. respectively of the protein N in unripe and ripe seeds of *P. sativum* var. *Greenfeast*, to be methionine N, and showed that there could have been very little free methionine in the seeds. On the other hand, although Almquist, Mecchi, Kratzer and Grau

(1942) considered methionine to be the growth-limiting deficiency in the protein of soya bean (Glycine soja), they estimated the methionine contents of raw soya bean protein and of soya bean meal protein to be 1·9 p.c. and 2·1 p.c. respectively, and whilst their failure to indicate protein purity by quoting the nitrogen contents makes it impossible to calculate the methionine contents in terms of the protein N, it is apparent that the values would be somewhat larger than any found by Lugg and Weller (1944) for other legume seeds.

Little information appears to have been obtained hitherto concerning the tyrosine and tryptophane contents of legume seeds and of their "whole" proteins. Virtanen and Laine (1936) estimated the tryptophane N in pea seeds (presumably *P. sativum* var.?) to be 1·06 p.c. of the total N, but their estimate may have been vitiated by the presence of other indoles in the seeds. Lugg and Weller (1941) found 1·70 p.c. tyrosine N and 0·62 p.c. tryptophane N in the "whole" protein of embryos of ripe seeds of *Lupinus angustifolius*, and pointed out that from the work of Bauguess and Berg (1934) it would have to be presumed that at a low level of intake the protein would not satisfy the tryptophane requirements of rats. Csonka and Jones (1936) found in various samples of defatted soya beans, from which most of the testas were removed, 1·90–2·60 p.c. of the nitrogen to be tyrosine N by the method of Folin and Ciocalteu (1927), and 1·35–1·77 p.c. to be tryptophane N by Csonka's (1932) modification of the May and Rose (1922) method. By his modified procedure, however, Csonka (1932) found 2·09 p.c. of tryptophane in casein. As explained later, we consider values of this order for the tryptophane content of casein to be much too large, and we are accordingly of the opinion that there may have been less tryptophane in the defatted soya beans than was estimated by Csonka and Jones (1936). Incidentally, Almquist, Mecchi, Kratzer and Grau (1942) found no appreciable tryptophane deficiency in heated soya bean protein for growth of chicks, when the protein comprised 20 p.c. of the food intake.

Rose's (1937) tentative estimate of the tryptophane requirement for normal growth of the rat as 0·2 p.c. of the food intake, is of similar magnitude to the estimate which may be made from the work of Bauguess and Berg (1934). The work of Totter and Berg (1939) suggests that a somewhat lower concentration may suffice for the mouse. Almquist and Mecchi (1941) considered that 0·5–0·6 p.c. of the food intake must be tryptophane if chicks are to show optimal growth, and this estimate is clearly indicated by their feeding trials with pure *l*-tryptophane. On the other hand, their experiments with whole casein, which they believed indicated that the requirement need not be above 0·44 p.c. of the food intake, involved calculations based upon a presumed tryptophane content of casein of 2·2 p.c. We consider this value to be some 72 p.c. too large (Lugg, 1938c), and in our opinion, therefore, the maximal requirement calculated from the results of the experiments with casein should be 0·26 p.c. of the food intake instead of 0·44 p.c.[3] The dietary requirement of tryptophane for sheep is not known. As with the cyst(e)ine and methionine requirements, the question of rumen synthesis is again involved.

The "whole" protein in the *L. luteus* embryos is of about the same tryptophane content as that in the embryos of *L. angustifolius*. The tryptophane content of the "whole" protein in the *T. subterrancum* seeds is higher, but is still rather low. In the light of the foregoing discussion it seems unlikely that these proteins, even when comprising 20 p.c. of the food intake, could provide sufficient tryptophane for optimal growth of rats and chicks. The tyrosine contents appear to be of moderate magnitude.

It is possible that legume seeds and their "whole" proteins are, in general, of rather low tryptophane content, as well as of low methionine content. But whereas a considerable body of evidence now exists to justify such a rough generalization concerning the methionine contents, the information concerning the trypto-

[3] Since this article went to press we have seen a further paper (Grau and Almquist, 1944: J. Nutrition, 28, p. 263), in which it is suggested that the *l*-tryptophane used in the earlier work may have been largely racemized. They find the requirement of *l*-tryptophane for the chick to be approximately half the value previously reported. This new value conforms with our calculation of the requirement based upon the feeding trials with casein.

phane contents is more meagre. Even so, the existing information suggests, in the absence of actual knowledge, that the tryptophane content of the "whole" protein in the seed of any legume, may be rather low.


## SUMMARY.

The ripe fructifications, or heads, of *Trifolium subterraneum* have been examined, with special regard to the sulphur distributions and to the cyst(e)ine and methionine contents of the "whole" proteins in the seeds and in the rest of the head material.

The seeds and their "whole" proteins were found to be of very low methionine content. They are thus in conformity with the general pattern indicated by Lugg and Weller (1944). They were found to be of very low cyst(e)ine content too.

The "whole" protein in the rest of the head material was found to be of very low methionine content and of moderate cyst(e)ine content.

The "whole" proteins of ripe seeds of *T. subterraneum* and of the embryos of ripe seeds of *Lupinus luteus* were found to be of moderate tyrosine content and rather low tryptophane content. Similar findings were made by Lugg and Weller (1941) for the "whole" protein of embryos of ripe seeds of *L. angustifolius*. The work suggests that the tryptophane contents of the "whole" proteins in the seeds of other legumes may be rather low.

The implications of these findings in regard to the nutrition of animals, have been discussed briefly.


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